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(54) Title: RECIPROCAL SUBTRACTION DIFFERENTIAL DISPLAY

(57) Abstract

This invention provides a method for identifying differentially expressed nucleic acids between two samples, comprising: a) selecting a first and second nucleic acid sample; b) producing libraries for the first and second nucleic acid sample; c) performing reciprocal subtraction between the libraries to produce two subtracted libraries; d) amplifying the two subtracted libraries; and e) comparing the two amplified subtracted libraries to identify differentially expressed nucleic acids. Also, this invention provides the above–described method, wherein the 3' primer used in the PCR amplification is an oligo dT 3' primer. This invention also provides the above–described methods, wherein the comparing of step e comprises using a gel to separate the nucleic acids from both of the libraries. This invention provides the isolated nucleic acid identified by the above–described methods, wherein the nucleic was not previously known to be differentially expressed between the two samples.

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RECIPROCAL SUBTRACTION DIFFERENTIAL DISPLAY

5   This application is a continuation-in-part of U.S. Serial
     No. 09/197,889, filed November 23, 1998, which is a
     continuation-in-part of U.S. Serial Application No.
     09/185,115, filed November 3, 1998 which is a
     continuation-in-part of U.S. Serial Application No.
10   09/032,684, filed February 27, 1998. The content of the
     above identified applications are hereby incorporated
     into this application by reference.

     Throughout this application, various references are
15   referred to within parentheses. Disclosures of these
     publications in their entireties are hereby incorporated
     by reference into this application to more fully describe
     the state of the art to which this invention pertains.

20   **Background of the Invention**
     Changes in gene expression are important determinants of
     normal cellular physiology, including cell cycle
     regulation, differentiation and development, and they
     directly contribute to abnormal cellular physiology,
25   including developmental anomalies, aberrant programs of
     differentiation and cancer (1-4). In these contexts,
     the identification, cloning and characterization of
     differentially expressed genes will provide relevant and
     important insights into the molecular determinants of
30   processes such as growth, development, aging,
     differentiation and cancer. A number of procedures can
     be used to identify and clone differentially expressed
     genes. These include, subtractive hybridization (5-10),
     differential RNA display (DDRT-PCR) (3,4, 11,12), RNA
35   fingerprinting by arbitrarily primed PCR (RAP-PCR)
     (13,14), representational difference analysis (RDA) (15),
     serial analysis of gene expression (SAGE) (16,17),
     electronic subtraction (18,19) and combinatorial gene
     matrix analyses (20).

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Since first introduced by Liang and Pardee (11), DDRT-PCR has gained wide popularity in analyzing and cloning differentially expressed genes. In DDRT-PCR, total RNAs or mRNAs from two or more cell types (or cells grown under different conditions, cells representing different stages of development, cells treated with agents modifying cellular physiology, etc.) are reverse-transcribed with two-base-pair anchored oligo dT primers, which divide mRNA populations into 12 cDNA subgroups. Then, each cDNA subgroup is amplified by PCR with one of 20 arbitrary 10-mer 5' primers and a 3' anchored primer and the PCR-amplified cDNA fragments are resolved in DNA sequencing gels. The combinations of primers are designed not only to yield a detectable size and number of bands, but also to display nearly the complete repertoire of mRNA species.

DDRT-PCR is a powerful methodology in which a vast number of mRNA species (>20,000, if no redundancy occurs) can be analyzed with only a small quantity of RNA (about 5 μg) (11). DDRT-PCR is often the method of choice when the RNA source is limiting, such as tissue biopsies. A direct advantage of DDRT-PCR is the ability to identify and isolate both up- and down-regulated differentially expressed genes in the same reaction. Furthermore, the DDRT-PCR technique permits the display of multiple samples in the same gel, which is useful in defining specific diagnostic alterations in RNA species and for temporally analyzing gene expression changes. However, the DDRT-PCR technique is not problem free. Difficulties encountered when using standard DDRT-PCR include, a high incidence of false positives and redundant gene identification, poor reproducibility, biased gene display and lack of functional information about the cloned cDNA. Furthermore, poor separation can mask differentially expressed genes of low abundance under the intense signals generated by highly expressed genes. The

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generation of false positives and redundancy can be highly problematic, resulting in an inordinate expenditure of resources to confirm appropriate differential expression and uniqueness of the isolated cDNAs. The cDNAs must be isolated from the gels in pure form (contamination of bands with multiple sequences complicates clone identification), reamplified, placed in an appropriate cloning vector, analyzed for authentic differential expression and finally sequenced. These limitations of the standard DDRT-PCR approaches emphasize the need for improvements in this procedure to more efficiently and selectively identify differentially expressed genes.

A number of modifications and improvements of the DDRT-PCR approach have been described (21-23). Single anchor or degenerate two-base anchor oligo dT primers can be used to streamline the massive numbers of reverse transcription and PCR reactions required for validation of cDNAs as well as to reduce false positives (24,25). Reproducibility can be improved by lengthening the arbitrary 5' primers to accommodate a convenient restriction site followed by two cycles of PCR with successive low- and high-stringency annealing temperatures (25,26). DDRT-PCR with inosine-containing 5' arbitrary primers can also increase reproducibility of this approach (27). However, since these modifications have only been analyzed using a subset of primers, further studies are necessary to validate these modifications of DDRT-PCR with additional primers and in several model systems.

In addition to genomic DNA contamination, mispriming, PCR artifacts, the high incidence of false positives and redundancy is also ascribed to poor separation between bands and the complexity of the templates amplified (28). Furthermore, poor separation can mask differentially

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expressed genes of low abundance under the intense signals generated by highly expressed genes. By enriching for unique cDNAs and removing common ones, it should in principle be possible to enrich for low abundant gene products and significantly decrease the complexity of amplified sequences. In addition, the sequence bias of DDRT-PCR should also be reduced by decreasing template complexity. These assumptions serve as the basis for the development of reciprocal subtraction differential RNA display (RSDD).

Subtractive hybridization, in which hybridization between tester and driver is followed by selective removal of common gene products, enriches for unique gene products in the tester cDNA population and reduces the abundance of common cDNAs (9). A subtracted cDNA library can be analyzed to identify and clone differentially expressed genes by randomly picking colonies or by differential screening (29-31). Although subtractive hybridization has been successfully used to clone a number of differentially expressed genes (5-7,10), this approach is both labor-intensive and does not result in isolation of the full spectrum of genes displaying altered expression (9,18).

In principle, DDRT-PCR performed with subtracted RNA or cDNA samples represents a powerful strategy to clone up and down-regulated gene products. This approach should result in the enrichment of unique sequences and a reduction or elimination of common sequences. This scheme should also result in a consistent reduction in band complexity on a display gel, thereby permitting a clearer separation of cDNAs resulting in fewer false positive reactions. Additionally, it should be possible to use fewer primer sets for reverse transcription and PCR reactions to analyze the complete spectrum of differentially expressed genes. Of particular importance

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for gene identification and isolation, rare gene products that are masked by strong common gene products should be displayed by using subtraction hybridization in combination with DDRT-PCR. In addition, the DDRT-PCR

5   approach with subtractive libraries could also prove valuable for efficiently screening subtracted cDNA libraries for differentially expressed genes. However, even though subtraction hybridization plus DDRT-PCR appears attractive for the reasons indicated above, a

10 previous attempt to use this approach has proven of only marginal success in consistently reducing the complexity of the signals generated, compared with the standard DDRT-PCR scheme (32).

15 We presently describe a reciprocal subtraction differential RNA display (RSDD) approach that efficiently and consistently reduces the complexity of DDRT-PCR and results in the identification and cloning of genes displaying anticipated differential expression.

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Summary of the Invention

This invention provides a method for identifying differentially expressed nucleic acids between two samples, comprising: (a) selecting a first and second nucleic acid sample, wherein the nucleic acid samples contain a repertoire of nucleic acids; (b) performing reciprocal subtraction between the nucleic acid samples to produce two subtracted nucleic acid samples; (c) amplifying the two subtracted nucleic acid samples; and (d) comparing the two subtracted nucleic acid samples to identify differentially expressed nucleic acids.

This invention also provides a method for identifying differentially expressed nucleic acids between two samples, comprising: (a) selecting a first and second nucleic acid sample, wherein the nucleic acid samples contain a repertoire of nucleic acids; (b) amplifying the two nucleic acid samples; (c) performing reciprocal subtraction between the amplified nucleic acid samples to produce two subtracted nucleic acid samples; and (d) comparing the two subtracted nucleic acid samples to identify differentially expressed nucleic acids.

This invention further provides the above-described methods, wherein the first and second nucleic acid samples are obtained from cells in different developmental stages.

This invention further provides the above-described methods, wherein the first and second nucleic acid samples are obtained from cells from different tissue types.

Also, this invention provides the above-described methods, wherein the 3' primer used in the PCR amplification is an oligo dT 3' primer.

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In addition, this invention provides the above-described methods, wherein the 3' primer used in the PCR amplification is a single anchor oligo dT 3' primer.

5   This invention also provides the above-described methods, wherein the comparing of step (e) comprises using a gel to separate the nucleic acids from both of the libraries.

This invention provides the isolated nucleic acid
10 identified by the the above-described methods, wherein the nucleic acid was not previously known to be differentially expressed between the two samples.

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Brief Description of the Figures

Figure 1
Identification of differentially expressed sequence tags
using reciprocal subtraction differential RNA display
(RSDD). Left panel: differential RNA display pattern of
conventional DDRT-PCR with RNA from E11 (C) and E11-NMT
(T) cells and an RSDD analysis of reciprocally subtracted
E11 minus E11-NMT (C/T) and E11-NMT minus E11 (T/C) cDNA
libraries. Right panel: representative RSDD patterns
using different sets of primers.

Figure 2
Reverse Northern analysis of differentially expressed
sequence tags identified by reciprocal subtraction
differential RNA display (RSDD). Differentially expressed
sequence tags obtained from RSDD were dot-blotted onto
Nylon membranes and probed with 32P-cDNA reverse
transcribed from RNA samples of E11 and E11-NMT cells.

Figure 3A
Differential expression of representative progression
elevated genes (PEGen) and progression suppressed genes
(PSGen) identified by reciprocal subtraction differential
RNA display (RSDD) and reverse Northern blotting.
Northern blots of E11 and E11-NMT RNA samples were probed
with radiolabeled (^{32}P) expressed sequence tags identified
by RSDD and reverse Northern blotting.

Figure 3B
Differential expression of representative progression
elevated genes (PEGen) and progression suppressed genes
(PSGen) identified by reciprocal subtraction differential
RNA display (RSDD) and reverse Northern blotting.

Figure 4
Differential expression of representative progression

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elevated genes (PEGen) and progression suppressed genes (PSGen) identified by reciprocal subtraction differential RNA display (RSDD) and reverse Northern blotting. Northern blots of cells displaying various stages of transformation progression were probed with radiolabeled (^{32}P) expressed sequence tags identified by RSDD and reverse Northern blotting. The cell types used include, Unprogressed E11 (-), CREFxE11-NMT F1 (-) and CREFxE11-NMT F2 (-) somatic cell hybrids, E11xE11-NMT A6 (-) somatic cell hybrid, E11xE11-NMT 3b (-) somatic cell hybrid, and E11-NMT Aza B1 (-) and E11-NMT Aza C1 (-) 5-azacytidine treated E11-NMT clones; and Progressed E11-NMT (+), CREFxE11-NMT R1 (+) and CREFxE11-NMT R2 (+) somatic cell hybrids, E11xE11-NMT A6TD (+) nude mouse tumor derived somatic cell hybrid, E11xE11-NMT IIa (+), E11-Ras R12 (+) a Ha-ras transformed E11 clone and E11-HPV E6/E7 (+) an E11 clone transformed by the E6 and E7 region of HPV-18.

Figure 5
cDNA fragment of PEGen 7 - 90% Homology to Human HPV16 E1BP. (Sequence ID No. 1)

Figure 6
cDNA fragment of PEGen 8 - Rat phosphofructose kinase C. (Sequence ID No. 2)

Figure 7
First (Sequence ID No. 3) and second (Sequence ID No. 4) cDNA fragments of PEGen 13.

Figure 8
cDNA fragment of PEGen 14. (Sequence ID No. 5)

Figure 9
cDNA fragment of PEGen 15. (Sequence ID No. 6)

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Figure 10
cDNA fragment of PEGen 21 which has 94% homology to mouse
FIN 14. (Sequence ID No. 7)

5   Figure 11
cDNA fragment of PEGen 24. (Sequence ID No. 8)

Figure 12
cDNA fragment of PEGen 26 - Rat poly ADP-ribose
10   polymerase. (Sequence ID No. 9)

Figure 13
cDNA fragment of PEGen 28. (Sequence ID No. 10)

15   Figure 14
cDNA fragment of PEGen 42. (Sequence ID No. 11)

Figure 15
cDNA fragment of PEGen 43. (Sequence ID No. 12)

20

Figure 16
cDNA fragment of PEGen 44. (Sequence ID No. 13)

Figure 17
25   cDNA fragment of PEGen 48. (Sequence ID No. 14)

Figure 18
cDNA fragment of PSGen 1 which has 80% homology to *B.
taurus* supervillin. (Sequence ID No. 15)

30

Figure 19
cDNA fragment of PSGen 2 which has 91% homology to human
HTLV-1 Tax interacting protein. (Sequence ID No. 16)

35   Figure 20
cDNA fragment of PSGen 4 - Rat proteasome activator.
(Sequence ID No. 17)

Figure 21
cDNA fragment of PSGen 10 - Rat Ferritin Heavy Chain.
(Sequence ID No. 18)

5   Figure 22
cDNA fragment of PSGen 12.   (Sequence ID No. 19)

Figure 23
cDNA fragment of PSGen 13.   (Sequence ID No. 20)

10
Figure 24
cDNA fragment of PSGen 23.   (Sequence ID No. 21)

Figure 25
15   cDNA fragment of PSGen 24.   (Sequence ID No. 22)

Figure 26
cDNA fragment of PSGen 25.   (Sequence ID No. 23)

20   Figure 27
cDNA fragment of PSGen 26.

Figure 28
cDNA fragment of PSGen 27.

25
Figure 29
cDNA fragment of PSGen 28.

Figure 30
30   cDNA fragment of PSGen 29.

Figure 31
cDNA fragment of PEGen 32.

35

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Figure 32

Schematic outline of the reciprocal differential RNA
display (RSDD) protocol. This scheme incorporates three
steps, reciprocal subtraction of cDNA libraries,
differential display of *in vivo* excised cDNAs and
expression analysis by reverse Northern and standard
Northern blotting. For the present application of RSDD,
reciprocal subtraction hybridization was performed using
libraries constructed from E11 and E11-NMT cells, i.e.,
E11 minus E11-NMT and E11-NMT minus E11. Differentially
expressed cDNAs identified on gels using differential RNA
were isolated, reamplified and analyzed for expression by
reverse Northern blotting. To confirm differential
expression cDNAs were analyzed using Northern blotting.

Figure 33

Differential expression of representative progression
elevated (PEGen) and progression suppressed genes (PSGen)
identified by RSDD and reverse Northern blotting.
Northern blots of E11 and E11-NMT RNA samples were probed
with radiolabeled (^{32}P) expressed sequence tags identified
by RSDD and reverse Northern blotting. Equal loading of
E11 and E11-NMT RNA is demonstrated by ethidium bromide
(EtBr)Staining .

Figure 34

Differential expression of representative PEGen and PSGen
genes identified by RSDD and reverse Northern blotting in
a large panel of rodent cells displaying differences in
transformation progression. Northern blots of cells
displaying various stages of transformation progression
were probed with radiolabeled (^{32}P) expressed sequence
tags identified by RSDD and reverse Northern blotting.
The cell types used include: Unprogressed E11 (-), CREF
X E11-NMT F1 (-) and CREF X E11-NMT F2 (-) somatic cell
hybrids, E11 X E11-NMT A6 (-) somatic cell hybrid, E11 X
E11-NMT 3b (-) somatic cell hybrid, and E11-NMT AZA B1

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(-) and E11-NMT AZA C1 (-) 5-azacytidine-treated E11-NMT clones; and Progressed E11-NMT (+), CREF X E11-NMT R1 (+) and CREF X E11-NMT R2 (+) somatic cell hybrids, E11 X E11-NMT A6TD (+) nude mouse tumor derived somatic cell
5   hybrid, E11 X E11-NMT IIa (+), E11-Ras R12 (+) and E11-HPV E6/E7 (+) an E11 clone transformed by the E6 and E7 region of HPV-18. Equal loading of RNAs is demonstrated by ethidium bromide (EtBr) staining.

10   Figure 35 A
     PSGen 12 cDNA Seqeunce and PSGen 12 Protein Sequence

     Figure 35 B
     PSGen 13 cDNA Sequence and PSGen 13 Protein Sequence

15
     Figure 35 C
     PEGen 28 cDNA Sequence and PEGen 28 Protein Sequence

     Figure 35D
20   PEGen 32 cDNA Sequence and PEGen 32 Protein Sequence

     Figure 35 E
     PEGen 42 cDNA Sequence and PEGen 42 Protein Sequence

25   Figure 35 F
     PEGen 45 cDNA Sequence

     Figure 35 G-1 and Figure 35 G-2
     PEGen 50 cDNA Sequence which are different parts of the
30   gene.

     Figure 36
     PSGen 27 - Novel

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Detailed Description of the Invention

This invention provides a method for identifying differentially expressed nucleic acids between two samples, comprising: (a) selecting a first and second nucleic acid sample, wherein the nucleic acid samples contain a repertoire of nucleic acids; (b) performing reciprocal subtraction between the nucleic acid samples to produce two subtracted nucleic acid samples; (c) amplifying the two subtracted nucleic acid samples; and (d) comparing the two subtracted nucleic acid samples to identify differentially expressed nucleic acids.

In an embodiment, the nucleic acid samples are mRNA or derived from mRNA. In another embodiment, the nucleic acid samples are total RNA. In another embodiment, the nucleic acid samples are cDNA. In another embodiment, the nucleic acid samples are a nucleic acid library.

In an embodiment, differentially expressed nucleic acids are expressed at different levels. In a further embodiment, one of the nucleic acids is not expressed. In a different embodiment, one of the nucleic acids is expressed in truncated form.

As used herein, reciprocal subtraction includes using nucleic acid sample A to subtract common nucleic acids from nucleic acid sample B (based on hybridization) and also using nucleic acid sample B to subtract common nucleic acids from nucleic sample A. In an embodiment, the complement of nucleic acid sample A is used to subtract nucleic acids from nucleic acid sample B and the complement of nucleic acid sample B is used to subtract nucleic acids from nucleic acid sample A. In a further embodiment, the RNA of nucleic acid sample A is used to subtract nucleic acids from nucleic acid sample B and the RNA of nucleic acid sample B is used to subtract nucleic acids from nucleic acid sample A. In yet another

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embodiment, the cDNA of nucleic acid sample A is used to subtract nucleic acids from nucleic acid sample B and the cDNA of nucleic acid sample B is used to subtract nucleic acids from nucleic acid sample A.

As used herein, methods of amplification include PCR and rolling circle replication.

A basic description of nucleic acid amplification is described in Mullis, U.S. Patent No. 4,683,202, which is incorporated herein by reference. The amplification reaction uses a template nucleic acid contained in a sample, two primer sequences and inducing agents. The extension product of one primer when hybridized to the second primer becomes a template for the production of a complementary extension product and vice versa, and the process is repeated as often as is necessary to produce a detectable amount of the sequence.

The inducing agent may be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes. Suitable enzymes for this purpose include, for example, *E.coli* DNA polymerase I, thermostable *Taq* DNA polymerase, Klenow fragment of *E.coli* DNA polymerase I, T4 DNA polymerase, other available DNA polymerases, reverse transcriptase and other enzymes which will facilitate combination of the nucleotides in the proper manner to form amplification products. The oligonucleotide primers can be synthesized by automated instruments sold by a variety of manufacturers or can be commercially prepared based upon the nucleic acid sequence of this invention.

This invention also provides a method for identifying differentially expressed nucleic acids between two samples, comprising: a) selecting a first and second nucleic acid sample; b) producing libraries for the first

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and second nucleic acid sample; c) amplifying the two libraries; d) performing reciprocal subtraction between the amplified libraries to produce two subtracted libraries; and e) comparing the two subtracted libraries

5   to identify differentially expressed nucleic acids.

This invention also provides a method for identifying differentially expressed nucleic acids between two samples, comprising: (a) selecting a first and second

10   nucleic acid sample, wherein the nucleic acid samples contain a repertoire of nucleic acids; (b) amplifying the two nucleic acid samples; (c) performing reciprocal subtraction between the amplified nucleic acid samples to produce two subtracted nucleic acid samples; and (d)

15   comparing the two subtracted nucleic acid samples to identify differentially expressed nucleic acids.

This invention also provides the above-described methods, wherein the two subtracted nucleic acid samples from step

20   c are amplified prior to the comparing of step d.

This invention also provides the above-described methods, wherein the each of the nucleic acid samples comprises a library of nucleic acids.

25

This invention also provides the above-described methods, wherein the nucleic acid samples are obtained from total cellular RNA purified by hybridization with oligo (dT).

30   This invention also provides the above-described methods, wherein the nucleic acid samples are obtained from total RNA from E11 and E11-NMT cells.

E11 is an adenovirus-transformed rat embryo cell line

35   that acquires an aggressive oncogenic progression phenotype when injected into athymic nude mice and reisolated in cell culture (E11-NMT).

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This invention further provides the above-described methods, wherein the first and second nucleic acid samples are obtained from cells in different developmental stages.

This invention further provides the above-described methods, wherein the first and second nucleic acid samples are obtained from cells from different tissue types.

This invention further provides the above-described methods, wherein the first and second nucleic acid samples are obtained from cells that differ in their exposure to external factors or in their gene expression.

In an embodiment, cells that differ in their exposure to external factors or in their gene expression includes any cells that may have different levels of gene expression, wherein some genes may not be expressed at all. In another embodiment, cells that differ in their exposure to external factors or in their gene expression includes any cells that are likely to have different levels of gene expression, wherein some genes may not be expressed at all. In still another embodiment, cells that differ in their exposure to external factors or in their gene expression includes any cell that has a phenotypically recognizable difference.

A short list of examples of cells that differ in their exposure to external factors or in their gene expression includes: cancerous versus normal cells, advanced cancer progression cells versus ealier cancer stage cells, diseased cells versus nondiseased cells, infected cells versus noninfected cells, later developmental stage cells versus earlier developmental stage cells, cells after DNA damage versus cells before DNA damage, senescent cells versus younger cells, cells induced by growth factors

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versus cells not induced by growth factors, cells in the process of neurodegeneration versus normal cells, and cells exposed to a chemotherapeutic agent versus normal cells.

As used herein, different tissues types include but are not limited to tissues containing: cells grown under or exposed to different conditions, cells in different stages of development, cells treated with agents modifying cellular physiology, and cells having different functions.

In an embodiment, cells at different stages of development are cells taken or analyzed at times differing by one or more hours in the development of the cell or organism.

Further, this invention provides the above-described methods, wherein the amplifying of step (d) comprises PCR amplification.

Also, this invention provides the above-described methods, wherein the 3' primer used in the PCR amplification is an oligo dT 3' primer. A few examples of oligo dT primers are T_{13}, $T_{13}A$, and $T_{13}GA$.

In addition, this invention provides the above-described methods, wherein the 3' primer used in the PCR amplification is a single anchor oligo dT 3' primer. Olgio dT 3' primers include $T_{13}A$, $T_{13}C$, and $T_{13}G$.

This invention provides the above-described methods, wherein the PCR amplification uses a set of random primers.

This invention provides the above-described methods, wherein the 5' primer is an arbitrary primer.

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This invention also provides the above-described methods, wherein the comparing of step (e) comprises using a gel to separate the nucleic acids from both of the substracted libraries.

5

In an embodiment, the gel is a polyacrylamide gel. In another embodiment, the gel is an agarose gel.

This invention further provides the above-described
10   methods, further comprising PCR amplifying the first and second nucleic acid samples.

This invention also provides the above-described methods, further comprising reamplifying differentially expressed
15   bands.

This invention also provides the above-described methods, further comprising reamplifying differentially expressed nucleic acid.

20

In one method of reamplifying differentially expressed bands, differentially amplified bands from plasmids of each subtracted library were marked with an 18G needle through the film and cut out with a razor. The cut out
25   differentially expressed bands can be reamplified (i.e. by PCR) and examined by reverse Northern and Northern blot analyses.

In addition, this invention provides the above-described
30   methods, wherein the comparing of step (e) comprises comparing the band intensities of the two amplified differentially expressed nucleic acids.

In addition, this invention provides the above-described
35   methods, wherein the nucleic acid samples are mRNA or cDNA derived from mRNA.

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In addition, this invention provides the above-described methods, wherein the comparing of step (e) comprises comparing the quantities of the two amplified differentially expressed nucleic acids.

5

This invention further provides the above-described methods, wherein the differences in band intensity between the two subtracted libraries are electronically quantified.

10

This invention further provides the above-described methods, wherein the differences in the quantities of nucleic acid between the two subtracted libraries are electronically quantified.

15

In one embodiment, electronic quantification involves using a scanner to detect the bands. In a further embodiment, computer software, such as Corel Draw, can be used to determine the pixel intensity of the scanned

20   image, thereby quantifying the band intensity.

Also, this invention provides the above-described methods, wherein the libraries of step (b) are constructed with λ-ZAP cDNA library kits. One skilled in

25   the art would recognize that any cDNA library would be suitable.

This invention provides the isolated nucleic acid identified by the the above-described methods, wherein

30   the nucleic acid was not previously known.

This invention also provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PSGen 12 (AI 144569).

35

In addition, this invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid

is the nucleic acid designated PSGen 13 (Accession No. AI 144570).

5   This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PSGen 23.

This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the

10   nucleic acid designated PSGen 24.

This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PSGen 25.

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This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PSGen 26 (Accession No. AI 144571).

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This invention also provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PSGen 27 (Accession No. AI 144572).

25

This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PSGen 28 (AI 144573).

30   This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PSGen 29 (AI 144574).

This invention provides the above-described isolated

35   nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 13 (AI 144564).

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This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 14 (AI 144565).

5   This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 15.

This invention provides the above-described isolated
10   nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 24 (Accession No. AI 144566).

This invention provides the above-described isolated
15   nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 28 (AI 144567).

This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the
20   nucleic acid designated PEGen 32 (AI 144568).

This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 42.

25
This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 43.

30   This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 44.

This invention provides the above-described isolated
35   nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 48.

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This invention further provides a previously unknown
isolated nucleic acid molecule identified by the above-
described methods which comprises (a) one of the nucleic
acid sequences as set forth in Figure 35; (b) a sequence
being degenerated to a sequence of (a) as a result of the
genetic code; (c) a sequence encoding one of the amino
acid sequences as set forth in Figure 35.(d) a sequence
of at least 12 nucleotides capable of specifically
hybridizing to the sequence of (a), (b) or (c).

Finally, this invention provides a purified polypeptide
comprising one of the amino acid sequence as set forth in
Figure 35.

The sequences of the cDNA of PSGen 12, PSGen 13, PSGen
26, PSGen 27, PSGen 28, PSGen 29, PEGen 13, PEGen 14,
PEGen 24, PEGen 28, and PEGen 32 were submitted to
GenBank and assigned with accession numbers AI 144569, AI
144570, AI 144571, AI 144572, AI 144573, AI 144574, AI
144564, AI 144565, AI 144566, AI 144567 and AI 144568
respectively.

This invention will be better understood from the
Experimental Details which follow. However, one skilled
in the art will readily appreciate that the specific
methods and results discussed are merely illustrative of
the invention as described more fully in the claims which
follow thereafter.

Experimental Details
We presently describe a reciprocal subtraction
differential RNA display (RSDD) approach that efficiently
and consistently reduces the complexity of DDRT-PCR and
results in the identification and cloning of genes
displaying anticipated differential expression. Proof of
principle for the RSDD approach has come from its
application for the identification of genes

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differentially expressed during cancer progression. RSDD
has resulted in the identification and cloning of genes
displaying elevated expression in progressed tumor cells
(PEGen) and reduced expression in progressed tumor cells

5   (PSGen).   The   model   used   for   RSDD   was   an
adenovirus-transformed rat embryo cell line, E11, that
acquires an aggressive oncogenic progression phenotype
when injected into athymic nude mice and reisolated in
cell culture (E11-NMT) (10,33,34).   Injection of E11

10   cells into nude mice results in tumors in 100% of animals
with a tumor latency time of approximately 35 to 40 days,
whereas E11-NMT cells form tumors in 100% of nude mice
with a tumor latency time of 15 to 20 days (10,34,35).
Additionally, E11 cells form colonies in agar with an

15   efficiency of ~3%, whereas E11-NMT display an agar
cloning efficiency of >30% (10,33,34).   The increased
tumorigenicity and enhanced anchorage independence
phenotypes are key indicators of tumor progression in the
E11/E11-NMT model system (10,33,34).

20

Differential RNA display was directly performed with
reciprocally subtracted cDNA plasmid libraries (E11 minus
E11-NMT and E11-NMT minus E11).   Compared with the
subtraction of PCR-amplified cDNA in Hakvoort et al., the

25   subtracted cDNA libraries used in this experiment are
free from potential PCR artifacts and provide more stable
and consistent sources for DDRT-PCR analyzes.   In
addition, three single anchored oligo dT 3' primers were
used instead of two-base-anchored approach described by

30   Hakvoort et al (32).   To further streamline the DDRT-PCR
procedure, reamplified cDNAs identified using RSDD were
analyzed using the reverse Northern blotting procedure
(35,36).   cDNAs displaying differential expression by
reverse Northern blotting were subsequently confirmed for

35   true differential expression by Northern analysis. These
modifications incorporated in the RSDD strategy result in
an efficient approach for using subtractive hybridization

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and DDRT-PCR for identifying differentially expressed
genes.

Methods

5   Total RNA from E11 and E11-NMT cells was isolated by the
guanidinium isothiocyanate/CsCl centrifugation procedure
and poly A^+ RNA was purified with oligo(dT) cellulose
chromatography (5). Two λ-ZAP cDNA libraries from E11 and
E11-NMT mRNA's were constructed with λ-ZAP cDNA library

10   Kits (Stratagene) following the manufacturer's protocol.
Reciprocal subtraction between E11 and E11-NMT libraries
was performed and two subtracted cDNA libraries (E11
minus E11-NMT and E11-NMT minus E11) were constructed as
described previously. Bacterial plasmid libraries from

15   the subtracted λ-ZAP cDNA libraries were obtained by *in
vivo* excision following the manufacturer's protocol
(Stratagene) and the plasmids were isolated with Qiagen
columns (Qiagen Inc.).

20   The purified plasmids of reciprocally subtracted cDNA
libraries were directly subjected to differential display
as in Liang et. al. (38) with minor modifications. The
plasmids of reciprocally subtracted cDNA libraries were
PCR-amplified with the combination of three single-anchor

25   3' primers (T $_{13}$ A, T $_{13}$ C or T $_{13}$ G) and 18 arbitrary 5'
10-mer primers obtained from Operon Technology Inc.
(Alameda, CA. OPA 1-20 except OPA1 and 3). The 20 μl PCR
reaction consisted of 10 mM Tris-HCl pH 8.4, 50 mM KCl,
1.5 mM MgCl$_2$, 2 μM each dNTP, 0.2 μM 5' arbitrary primer,

30   1 μM 3' anchor primer, 50 ng of plasmid of a subtracted
library, 10 μCi α-^{35}S-dATP (3000 Ci/mmole from Amersham)
and 1 U of Taq DNA polymerase (Gibco BRL). The
parameters of PCR were 30 sec at 95 C, 40 cycle of 30 sec
at 95 C, 2 min. at 40 C and 30 sec at 72 C and additional

35   5 min. at 72 C. After the cycling, 10 μl of 95%
formamide, 0.05% bromophenol blue and 0.05% xylene cyanol
were added to each PCR reaction. The mixture was heated

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at 95 °C for 2 min and separated in a 5% denaturing DNA sequencing gel maintained at 50 °C. PCR reactions of plasmids from each subtracted library in a primer set were run side by side. Differentially amplified bands from plasmids of each subtracted library were marked with an 18G needle through the film and cut out with a razor. The gel slice was put in 100 μl TE pH 8.0 and incubated at 4 °C overnight. After the incubation, the mixture was boiled for 5 min and microcentrifuged for two min. The supernatant was collected and stored at -20 °C until reamplification. The band extract was reamplified with the same cycling parameters in a 50 μl reaction consisting of 10 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl$_2$, 20 μM each dNTP, 0.2 μM 5' arbitrary primer, 1 μM 3' anchor primer, 5 μl of band extract and 2.5 U of Taq DNA polymerase (Gibco BRL).

Differential expression of the reamplified DNA fragment was scrutinized by reverse Northern and Northern blot analyses. In reverse Northern analysis, after confirmation in a 1% agarose gel, the reamplified DNA fragment (10 μl of PCR reaction) was mixed with 90 μl TE and spotted on a positively charged Nylon membrane (Boehringer Mannheim) with a 96-well vacuum manifold. The membrane was soaked with denaturing and neutralizing solution successively, and the spotted DNA was crosslinked to the membrane with a UV crosslinker (Stratagene). ^{32}P-labeled first strand cDNA was prepared by reverse transcription of total RNA. After heating at 70 °C for 10 min and quenching on ice for two min, 0.4 μM each T$_{13}$A, T$_{13}$G and T$_{13}$C and 10 μg total RNA mixture was added with 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dTTP, 0.02 mM dCTP, 0.5 μl RNase inhibitor (Gibco BRL), 100 μCi dCTP (3000 Ci/mmole from Amersham) and 200 U Superscript RT II (Gibco BRL) in a final 25 μl reaction. The reaction mixture was incubated at 42 °C for one hr and at

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37 °C for 30 min after addition of 2 μl of RNase H (10U, Gibco BRL). The membrane was hybridized at 42 °C overnight in a 50% formamide hybridization solution. The hybridized membrane was washed at room temperature for 15

5   min with 2X SSC containing 0.1% SDS twice and at 55 °C for at least one hr with 0.1X SSC containing 0.1% SDS, successively. The membrane was probed with the ^{32}P-labeled cDNA of E11, stripped off and probed with ^{32}P-labeled cDNA of E11-NMT. The signal intensity of each

10   spot was normalized against that of GAPDH and compared between E11 and E11-NMT. Reamplified DNA fragments displaying differential expression levels ≥1.8-fold higher between the two cell types were selected and analyzed by Northern blotting analysis.

15

In Northern blot analysis, 10 μg of total RNA from E11 and E11-NMT cells were run side-by-side in a 1% agarose gel with formaldehyde and transferred to a positively charged Nylon membrane. Reamplification reaction (5 μl)

20   was ^{32}P-labeled with a multiprime labeling kit (Boehringer Mannheim) used to probe the membrane as described above. DNA fragments expressed differentially between E11 and E11-NMT in Northern blot analyses were cloned into the Eco RV site of the pZEro-2.1 cloning vector (Invitrogene)

25   and sequenced. In order to confirm differential expression, the cloned cDNA fragment was released by Eco RI -Xho I, ^{32}P-labeled and used to probe Northern blots as described above. Samples of RNAs from various E11 and E11-NMT derivatives displaying either a progressed or

30   suppressed progression phenotype, based on nude mice tumorigenesis and soft agar cloning assays were analyzed. These included E11, E11-NMT, CREF X E11-NMT F1 and F2 somatic cell hybrids (suppressed progression phenotype), CREF X E11-NMT R1 and R2 somatic cell hybrids

35   (progression phenotype), E11 X E11-NMT A6 somatic cell hybrid (suppressed progression phenotype), E11 X E11-NMT A6TD tumor-derived somatic cell hybrid (progression

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phenotype), E11 X E11-NMT 3b somatic cell hybrid (suppressed progression phenotype), E11 X E11-NMT 2a (progression phenotype), E11-NMT AZA B1 and C1 5-azacytidine treated E11-NMT clones (suppressed progression phenotype), E11-ras R12 clone containing the Ha-ras oncogene (progression phenotype) and E11-HPV E6/E7 clone containing the human papilloma virus-18 E6 and E7 gene region (progression phenotype). Differential expression of the PEGen and PSGen genes in the various cell types was confirmed using ^{32}P-labeled probes and Northern hybridization analysis. After reconfirmation of differential expression, the plasmids containing the differentially expressed DNA fragments were sequenced by the dideoxy sequencing procedure.

Results and Discussion

Subtraction hybridization provides a direct means of enriching for unique cDNA species and eliminating common sequences between complex genomes. DDRT-PCR is a proven methodology for the rapid identification and cloning of differentially expressed sequences between cell types (3,4,22). In principle, subtraction hybridization combined with DDRT-PCR should reduce band complexity which often obscures the identification of differentially expressed genes and generates false positive signals (23,28). This strategy, RSDD, has been used to analyze genes differentially expressed during transformation progression. The differential RNA display pattern of E11 and E11-NMT cells using standard differential RNA display DDRT-PCR) and RSDD is shown in Fig. 1 (Left Panel). As predicted, the differential RNA display pattern of RSDD was much less complex than that of DDRT-PCR. The majority of bands common to both cDNA samples were eliminated using RSDD. These experiments demonstrate that subtractive hybridization prior to differential RNA display is effective in simplifying display patterns

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permits the efficient identification of differentially expressed cDNAs. Since RSDD significantly reduced the number of bands displayed, single anchor oligo dT primers, that can increase band numbers, were

5   successfully used in subsequent applications of the RSDD approach (Fig. 1; Right Panel). Using RSDD, 235 differentially displayed cDNAs in the E11/E11-NMT tumor progression model system were isolated.

10   Hakvoort et. al. (32) used a reciprocal subtraction approach to analyze gene expression changes resulting during liver regeneration following 70% hepatectomy, i.e., normal liver subtracted from partially hepatectomized regenerating liver and vice versa.

15   Although some bands displayed apparent enrichment, the complexity of the display pattern did not show appreciable simplification. These results are in stark contrast to RSDD, which results in a clear delineation and simplification of differentially expressed amplified

20   bands (Figs. 1). Although conceptually similar, RSDD is significantly more effective than the subtraction plus DDRT-PCR approach described by Hakvoort et al. (32). The improved efficiency of RSDD versus the Hakvoort et al. (32) approach can be attributed to several factors. The

25   approach of Hakvoort et al. (32) is based on the subtraction procedure described by Wang and Brown (38). This approach involves multiple rounds of PCR-amplification prior to each round of subtractive hybridization. In contrast, RSDD involves a single round

30   of reciprocal subtraction that does not involve PCR amplification (5,10). In this respect, the complicated display pattern observed by Hakvoort et al. (32) even after three or four rounds of subtraction might result from reduced subtraction efficiency, PCR artifacts or a

35   combination of these problems. Increasing the number of reactions by using two-base pair anchored oligo dT primers did not reduce the complexity of displayed bands

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(32). In these contexts, a critical component for the successful use of RSDD involves the use of an appropriate subtraction hybridization protocol, that can efficiently reduce cDNA complexity and generate stable populations of
5   cDNAs for analysis.

Previous studies demonstrate that different gene cloning strategies, including DDRT-PCR, subtraction hybridization and electronic display, identify dissimilar
10 differentially expressed genes (18). These results suggest that a single approach for gene identification may not identify the complete spectrum of differentially expressed genes (18). Similarly, RSDD and DDRT-PCR do not resolve the same differentially expressed bands (Fig.
15 1). Unique bands identified in DDRT-PCR that were differentially expressed when analyzed by Northern blotting were not the same as those found using RSDD and vise versa. These results are not surprising, since, as indicated above, subtraction hybridization and
20 differential RNA display identified distinct differentially expressed genes. Apparently, specific differentially expressed genes are lost during subtraction hybridization and differential RNA display of subtracted cDNAs. On the basis of these considerations,
25 it will be essential to use multiple gene discovery approaches to identify and clone the complete spectrum of differentially expressed genes.

DDRT-PCR can generate large numbers of differentially
30 displayed bands making subsequent analysis both labor intensive and a daunting challenge. In order to reduce these limitations of DDRT-PCR, RSDD has been used in combination with reverse Northern analyses of isolated cDNAs. Gel extracted cDNA fragments were reamplified,
35 dot-blotted on Nylon membranes and successively probed with reverse transcribed ^{32}P-cDNA from E11 or E11-NMT RNAs (Fig. 2). Signals were detected in 181 reamplified bands

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out of 235 (77%). This number is lower than that observed using DDRT-PCR (51 out of 54). However, this comparison may not be accurate since only four arbitrary primers were used for DDRT-PCR and fewer differentially

5   expressed bands were detected and isolated. A possible reason for the high incidence of false positives in RSDD may be due to the existence of foreign plasmid-like DNA in the cDNAs and the inaccurate reading properties of DDRT-PCR.

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Table 1. Differentially Expressed cDNA Fragments
Cloned by DDRT-PCR.

Nomenclature	Identity	Homology
PEGen 41	To be determined	
PEGen 42	Novel	Novel
PEGen 43	Novel	Novel
PEGen 44	Novel	Novel
PEGen 45	Hoxa11 locus antisense	mouse 90%
PEGen 46	Glutamyl t-RNA synthetase	human 59%
PEGen 48	Novel	Novel
PEGen 50	Novel	Novel
PSGen 1	Supervillin	*B. taurus 80%*
PSGen 2	HTLV-1 Tax interacting protein	human 91%
PSGen 4	Proteasome activator	Rat 100%
PSGen 27	Novel	

The signal intensities of the various cDNAs in reverse
Northern analysis were quantified and normalized against
that of GAPDH, which remained unchanged in E11 and
E11-NMT cells. The PEG-3 (PEGen-3) gene (10) was used as
an additional control, to verify increased expression in
E11-NMT versus E11 cells. In the reverse Northern

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analyses, PEGen-3 levels were 4-fold higher in E11-NMT than in E11 cells, which coincided with Northern blotting results, thereby demonstrating the concordance of reverse Northern and Northern assays. A ≥ 1.8-fold differential

5   cut-off (after normalization for GAPDH expression) was used to identify and isolate cDNA bands displaying modified expression in E11 versus E11-NMT cells. This resulted in the identification of 7 cDNAs with higher expression in E11 versus E11-NMT cells and 65 cDNAs with

10   elevated expression in E11-NMT versus E11 cells. These results suggest that tumor progression in E11-NMT cells correlates with the increased expression of a large number of genes, whereas only a smaller subset of genes display decreased expression.

15

A problem present in DDRT-PCR, that is reduced but still can occur in RSDD, is the isolation of multiple cDNA species from what appears to be a single amplified band. When this occurs, these multiple species can produce

20   spurious results when analyzed by reverse Northern analyses. For example, if two distinct species are isolated, one displaying modified expression and a second not displaying modified expression, an accurate estimate of differential expression will not be obtained by

25   reverse Northern analysis. In this case, a number of potential false positives generated using reverse Northern analyses, may in reality not be false positives, but instead may represent multiple cDNAs. This problem may be ameliorated by performing single strand

30   conformational polymorphism (SSCP) or reverse Northern analyses using cloned cDNA populations (39,40).

The expression pattern of representative RSDD-derived cDNAs in E11 versus E11-NMT and in a more expanded

35   E11/E11-NMT progression cell culture series is shown in Figs. 3 and 4, respectively. Reverse Northern results correlated well with Northern blots using E11 and

E11-NMT (~80% concordance) or a larger panel of cells differentially displaying the progression phenotype, including progression negative, E11, CREF x E11-NMT F1, CREF X E11-NMT F2, E11 X E11-NMT A6, E11 X E11-NMT 3b, E11-NMT Aza B1 and E11-NMT Aza C1, and progression positive E11-NMT, CREF X E11-NMT R1, CREF X E11-NMT R2, E11 X E11-NMT A6TD, E11 X E11-NMT IIa, E11-ras and E11-HPV E6/E7. Sequence analysis of the various progression upregulated genes (PEGen) and progression suppressed genes (PSGen) identified both known and unknown genes (Table 2). Known PEGen genes included PEGen 7 (HPV16 E1BP), PEGen 8 (PFK-C), PEGen 21 (FIN 14) and PEGen 26 (poly ADP-ribose polymerase) and a known PSGen gene was PSGen 10 (ferritin heavy chain). Two PEGen genes out of six were found to be novel (PEGen 14 and PEGen 24) and two PSGen genes out of three were found to be novel (PSGen 12 and PSGen 13) (Table 2).

Table 2.   Differentially Expressed cDNA Fragments
           Cloned by RSDD

	Nomenclature	Identity	Homology
5	PEGen 7	HPV16 E1BP	Human 90%
	PEGen 8	PFK-C	Rat 100%
10	PEGen 13	Novel	Novel
	PEGen 14	Novel	Novel
	PEGen 15	Novel	Novel
15	PEGen 21	FIN 14	Mouse 94%
	PEGen 24	Novel	Novel
20	PEGen 26	Poly ADP-ribose Polymerase	Rat 100%
	PEGen 28	Novel	Novel
25	PEGen 32	Novel	Novel
	PSGen 10	Ferritin Heavy Chain	Rat 100%
	PSGen 12	Novel	Novel
30	PSGen 13	Novel	Novel
	PSGen 23	Novel	Novel

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PSGen 24	Novel	Novel
PSGen 25	Novel	Novel
PSGen 26	Novel	Novel
PSGen 27	Novel	Novel
PSGen 28	Novel	Novel
PSGen 29	Novel	Novel

PEGen 7 is expressed at ~ 5-fold higher levels in E11-NMT than in E11 cells. PEGen 7 is ~90% homologous to 16E1-BP, a cDNA encoding a protein identified using the yeast two-hybrid assay that interacts with human papillomavirus type 16 E1 protein (41). 16E1-BP encodes a 432aa protein of unknown function but does contain an ATPase signature motif (Gly-X$_4$-Gly consensus ATP binding motif at aa 179 through 186). 16E1-BP appears to be a form of TRIP13, a protein previously shown to bind thyroid hormone receptor in yeast two-hybrid assays. The role of PEGen 7/16E1-BP in the progression phenotype in the E11/E11-NMT progression model is not known. Additional studies are necessary to determine if this gene change is associative or causative of transformation progression.

PEGen 8 is expressed at ~3- to 4- fold higher levels in E11-NMT than in E11 cells. PEGen 8 shows 100% homology to rat phosphofructokinase C (PFK-C) (42). PFK catalyzes the rate-limiting and committed step in glycolysis, the conversion of fructose 6-phosphate to fructose 1,6-biphosphate. Three subunit isozymes of PFK have been identified, that form homo- and heterotetramers with differing catalytic and allosteric properties. PFK-M is

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specific for cardiac and skeletal muscle, PFK-L is expressed in many tissues but is most abundant in the liver and PFK-C is expressed in several brain regions and the anterior pituitary but not in liver, skeletal muscle,

5   or several other human tissues. The cDNA of PFK-C isolated from a rat hypothalamic cDNA library is 2643 bp and encodes a protein of 765aa (42). In a recent study, Sanchez-Martinez and Aragon (43) demonstrated that PFK-C is the predominant form of PFK in ascites tumor cells

10   (obtained from a transplantable mouse carcinoma of mammary origin), whereas PFK-L is most abundant in the normal mammary gland. These results suggest the interesting possibility that PFK-C might contribute to the malignant nature of specific target cells. The role

15   of PEGen 8/PFK-C in progression in the E11/E11-NMT model remains to be determined.

PEGen 21 is expressed at ~3- to 4-fold higher levels in E11-NMT than in E11 cells. PEGen 21 displays ~94%

20   homology with the fibroblast growth factor-4 inducible gene FIN-14 (44). FIN-14 is a novel cDNA of unknown function that hybridizes with a 4.5 kb mRNA that is induced 4-fold in NIH3T3 mouse cells following treatment with FGF-4. The induction of FIN-14 occurs late (18 hr)

25   after treatment with FGF-4 and does not occur when cells are treated for 18 hr with FGF-4 in the presence of cycloheximide (44). These results confirm that FIN-14 encodes a late-inducible gene. Moreover, nuclear run-on assays document that FIN-14 is trancriptionally activated

30   in NIH3T3 cells following growth factor stimulation. Tissue distribution studies indicate expression of a single mRNA species in the kidney with low levels of expression observed in several other tissues including testis and thymus. Mouse embryogenesis studies indicate

35   that FIN-14 expression occurs constitutively in mouse embryos between day 10.5 and 15.5. Unlike NIH3T3, FIN-14 was constitutively expressed in PC12 cells and its level

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did not vary appreciably in response to growth factor
stimulation. The role of PEGen 21/FIN-14 in progression
in E11/E11-NMT model system is not currently known.

5   The PSGen cDNAs, PSGen-12 and PSGen-13, consist of
sequences without homology to those presently reported in
various DNA databases. Expression of these cDNAs is ~3-
to 4-fold higher in E11 versus E11-NMT cells (Fig. 3).
It is not currently known whether these genes simply
10   correlate with or functionally regulate the progression
phenotype. The identification of full-length cDNAs for
·   PSGen-12 and PSGen-13 are in progress and once identified
experiments can be conducted to directly define the role
of these PSGen's in cancer progression.

15

We presently demonstrate that a modified differential RNA
display technique, RSDD, can efficiently identify
differentially expressed cDNAs. As predicted,
subtractive hybridization prior to differential RNA
20   display greatly reduces band complexity, a problem
encountered in standard DDRT-PCR in which RNA samples are
directly analyzed without subtraction. Unlike a previous
report using subtracted cDNAs processed through
successive rounds of PCR (32,45), common bands were
25   eliminated using reciprocally subtracted cDNA libraries
that had not been processed using PCR. In addition to
subtraction hybridization, the discovery of
differentially expressed genes was further streamlined by
using reverse Northern analyses with isolated cDNAs.
30   With 3 single anchored oligo dT primers and 18 arbitrary
5' primers, 72 bands were identified that displayed
differential expression using reverse Northern analysis.
Currently, 40 of these cDNA species have been analyzed by
Northern blotting and found to display differential
35   expression in E11 versus E11-NMT cells. Subsequent
studies with the majority of these RSDD cDNAs
demonstrated coordinated expression with the progression

phenotype in a large panel of unprogressed and progressed transformed cells. Current sequence analysis of the cloned cDNA fragments revealed 9 different genes, including 4 novel genes not reported in recent DNA

5   databases. RSDD represents a method of choice either as a more efficient and less time consuming modification of the differential RNA display strategy or as a screening methodology for identifying differentially expressed genes in reciprocally subtracted cDNA libraries.

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Second Series of Experiments

Presently described is a RSDD approach that efficiently
and consistently reduces the complexity of DDRT-PCR and
5   results in the identification and cloning of genes
displaying anticipated differential expression. The model
used for RSDD was an adenovirus-transformed rat embryo
cell line, E11, that acquires an aggressive oncogenic
progression phenotype when injected into athymic nude
10 mice   and   reestablished   in   cell   culture
(E11-NMT)(6,26,27). Injection of E11 cells into nude mice
results in tumors in 100% of animals with a tumor latency
time of approximately 35 to 40 days, whereas E11-NMT
cells form tumors in 100% of nude mice with a tumor
15 latency time of 15 to 20 days (6,26,27). Additionally,
E11 cells form colonies in agar with an efficiency of ~3
%, whereas E11-NMT display an agar cloning efficiency of
>30% (6,26,27). The increased tumorigenicity and enhanced
anchorage independence phenotypes are key indicators of
20 tumor progression in the E11/E11-NMT model system
(6,26,27). RSDD has resulted in the identification and
cloning of genes displaying elevated expression in
progressed tumor cells (progression elevated gene, PEGen)
and suppressed expression in progressed tumor cells
25 (progression suppressed gene, PSGen).

MATERIALS AND METHODS

RNA isolation and cDNA library construction. Total RNA
from E11 and E11-NMT cells was isolated by the
30 guanidinium isothiocyanate/CsCl centrifugation procedure
and poly(A)$^+$ RNA was purified with oligo(dT) cellulose
chromatography(5). Two λ-ZAP cDNA libraries from E11 and
E11-NMT mRNAs were constructed with λ-ZAP cDNA library
kits (Stratagene) following the manufacturer's protocol.
35 Reciprocal subtraction between E11 and E11-NMT libraries
was performed and two subtracted cDNA libraries (E11
minus E11-NMT and E11-NMT minus E11) were constructed as

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described(5,6). Plasmid cDNA libraries from the subtracted λ-ZAP cDNA libraries were obtained by in vivo excision following the manufacturer's protocol (Stratagene) and the plasmids were isolated with Qiagen
5   columns (Qiagen, Chatsworth, CA.).

RSDD methodology. The purified plasmids of reciprocally subtracted cDNA libraries were directly subjected to differential display as in Liang et al. (28) with minor
10   modifications. The plasmids of reciprocally subtracted cDNA libraries were PCR-amplified with the combination of three single-anchor 3' primers ($T_{13}A$, $T_{13}C$ or $T_{13}G$) and 18 arbitrary 5' 10-mer primers obtained from Operon Technology Inc. (Alameda, CA. OPA 1-20 except OPA1 and
15   3). The 20 μl PCR reaction consisted of 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 2 μM each dNTP, 0.2 μM 5' arbitrary primer, 1 μM 3' anchor primer, 50 ng of plasmid of a subtracted library, 10 μCi α-^{35}S-dATP (3,000 Ci/mmol from Amersham) and 1 unit of Taq DNA polymerase
20   (Gibco/BRL). The parameters of PCR were 30 sec at 95°C, 40 cycles of 30 sec at 95°C, 2 min at 40°C and 30 sec at 72°C and additional 5 min. at 72°C. After the cycling, 10 μl of 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol were added to each PCR reaction. The mixture was
25   heated at 95°C for 2 min and separated in a 5% denaturing DNA sequencing gel maintained at 50°C. PCR reactions of plasmids from each subtracted library in a primer set were run side by side. Differentially amplified bands from plasmids of each subtracted library were marked with
30   18G needle through the film and cut out with a razor. The gel slice was put in 100 μl TE (pH 8.0) and incubated at 4°C overnight. After the incubation, the mixture was boiled for 5 min and microcentrifuged for two min. The supernatant was collected and stored at -20°C until
35   reamplification. The band extract was reamplified with the same cycling parameters in a 50 μl reaction consisting of 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM

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MgCl$_2$, 20 μM each dNTP, 0.2 μM 5' arbitrary primer, 1 μM 3' anchor primer, 5μl of band extract and 2.5 units of Taq DNA polymerase (Gibco/BRL).

5   **Reverse Northern Blotting Procedure.** Differential expression of the reamplified DNA fragment was scrutinized by reverse Northern and Northern blot analyses. In reverse Northern analysis, after confirmation in a 1% agarose gel, the reamplified DNA

10   fragment (10 μl of PCR reaction) was mixed with 90 μl TE and spotted on a positively charged Nylon membrane (Boehringer Mannheim) with a 96-well vacuum manifold. The membrane was soaked with denaturing and neutralizing solution successively, and the spotted DNA was

15   crosslinked to the membrane with a UV crosslinker (Stratagene). ^{32}P-labeled first strand cDNA was prepared by reverse transcription of total RNA. After heating at 70°C for 10 min and quenching on ice for two min, 0.4 μM each T$_{13}$A, T$_{13}$G and T$_{13}$C and 10 μg total RNA mixture was

20   added with 50 mM Tris-HCl, (pH 8.3), 75 mM KCl, 3 mM MgCl$_2$, 10 mM DTT, 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dTTP, 0.02 mM dCTP, 0.5 μl RNase inhibitor (Gibco/BRL), 100 μCi dCTP (3,000 Ci/mmol from Amersham) and 200 units Superscript RT II (Gibco/BRL) in a final 25 μl reaction.

25   The reaction mixture was incubated at 42°C for one hour and at 37°C for 30 min after addition of 2 μl of RNase H (10 units, Gibco/BRL). The membrane was hybridized at 42°C overnight in a 50% formamide hybridization solution. The hybridized membrane was washed at room temperature for 15

30   min with 2X standard saline citrate containing 0.1% SDS twice and at 55°C for at least one hour with 0.1X Standard Saline Citrate containing 0.1% SDS, successively. The membrane was probed with the ^{32}P-labeled cDNA of E11, striped off and probed with ^{32}P-labeled cDNA of E11-NMT.

35   The signal intensity of each spot was normalized against that of glyceraldehyde-3-phosphate dehydrogenase and compared between E11 and E11-NMT. Reamplified DNA

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fragments displaying differential expression levels ≥1.8-fold higher between the two cell types were selected and analyzed by Northern bloting analysis.

5   **Northern Blotting Analysis.** In Northern blot analysis, 10 µg of total RNA from E11 and E11-NMT cells were run side-by-side in a 1% agarose gel with formaldehyde and transferred to a positively charged Nylon membrane. Reamplification reaction (5 µl) was ^{32}P-labeled with a
10   multiprime labeling kit (Boehringer Mannheim) used to probe the membrane as described above. DNA fragments expressed differentially between E11 and E11-NMT in Northern blot analyses were cloned into the EcoRV site of the pZEro-2.1 cloning vector (Invitrogene) and sequenced.
15

To confirm differential expression, the cloned cDNA fragment was released by EcoRI-XhoI, ^{32}P-labeled and used to probe Northern blots as described above. Samples of RNAs from various E11 and E11-NMT derivatives displaying
20   either a progressed or suppressed progression phenotype, based on nude mice tumorigenesis and soft agar cloning assays were analyzed. These included E11, E11-NMT, CREF x E11-NMT F1 and F2 somatic cell hybrids (suppressed progression phenotype), CREF x E11-NMT R1 and R2 somatic
25   cell hybrids (progression phenotype), E11 x E11-NMT A6 somatic cell hybrid (suppressed progression phenotype), E11 x E11-NMT A6TD tumor-derived somatic cell hybrid (progression phenotype), E11 x E11-NMT 3b somatic cell hybrid (suppressed progression phenotype), E11 x E11-NMT
30   IIa (progression phenotype), E11-NMT AZA B1 and C1 5-azacytidine treated E11-NMT clones (suppressed progression phenotype), E11-Ras R12 clone containing the Ha-ras oncogene (progression phenotype) and E11-HPV E6/E7 clone containing the human papilloma virus-18 E6 and E7
35   gene region (progression phenotype). Differential expression of the PEGen and PSGen genes in the various cell types was confirmed using ^{32}P-labeled probes and

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northern hybridization analysis. After reconfirmation of
differential expression, the plasmids containing the
differentially expressed DNA fragments were sequenced by
the dideoxy sequencing procedure.

5

RESULTS AND DISCUSSION

Subtraction hybridization provides a direct means of
enriching for unique cDNA species and eliminating common
sequences between complex genomes(7,18). DDRT-PCR is a
10   proven methodology for the rapid identification and
cloning of differentially expressed sequences between
cell   types   (1,2,28).   In   principle,   subtraction
hybridization combined with DDRT-PCR should reduce band
complexity which often obscures the identification of
15   differentially   expressed   genes   and   generates   false
positive signals (21,29). RSDD has been used to analyze
genes   differentially   expressed   during   transformation
progression (Fig. 28). Differential RNA display was
directly performed with reciprocally subtracted cDNA
20   plasmid libraries (E11 minus E11-NMT and E11-NMT minus
E11) that had not been subjected to PCR. Three single
anchored oligo dT 3' primers were used for subsequent
amplification prior to display. To further streamline the
DDRT-PCR procedure, reamplified cDNAs identified using
25   RSDD were analyzed using the reverse Northern blotting
procedure   (30,31).   cDNAs   displaying   differential
expression by reverse Northern blotting were subsequently
confirmed for true differential expression by Northern
analysis.

30

The differential RNA display pattern of E11 and E11-NMT
cells using standard differential RNA display (DDRT-PCR)
and RSDD is shown in Fig. 1 (Left Panel). The
differential RNA display pattern of RSDD is much less
35   complex than that of DDRT-PCR. These experiments
demonstrate that subtractive hybridization prior to
differential RNA display is effective in simplifying

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display patterns permitting the efficient identification
of differentially expressed cDNAs. Since RSDD
significantly reduced the number of bands displayed,
single anchor oligo dT primers, that can increase band
5 numbers, were successfully used in subsequent
applications of the RSDD approach (Fig. 1; Right Panel).
Using RSDD, 234 differentially displayed cDNAs in the
E11/E11-NMT tumor progression model system were isolated.
Hakvoort et al.(25) used a reciprocal subtraction
10 approach to analyze gene expression changes resulting
during liver regeneration following 70% hepatectomy,
i.e., normal liver subtracted from partially
hepatectomized regenerating liver and vice versa.
Although some bands displayed apparent enrichment, the
15 complexity of the display pattern did not show
appreciable simplification. In contrast, RSDD results in
a clearer delineation and simplification of
differentially expressed amplified bands (Figs. 1).
Although conceptually similar, RSDD is significantly more
20 effective than the subtraction plus DDRT-PCR approach
described by Hakvoort et al. (25) The reasons for the
improved efficiency of RSDD versus the Hakvoort et al.
(25) approach are not known. One possibility is that the
differences between the experimental approaches may
25 reflect the subtraction hybridization strategies
employed. The approach of Hakvoort et al. (25) is based
on the subtraction procedure described by Wang and Brown
(32). This approach uses multiple rounds of
PCR-amplification prior to each round of subtractive
30 hybridization. In contrast, RSDD involves a single round
of reciprocal subtraction without intermediate
amplification(5,6). In this respect, the complicated
display pattern observed by Hakvoort et al. (25) even
after three or four rounds of subtraction might result
35 from reduced subtraction efficiency, PCR artifacts or a
combination of these problems. Increasing the number of
reactions by using two-base pair anchored oligo dT

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primers did not reduce the complexity of displayed bands
(25). In these contexts, a critical component for the
successful use of RSDD involves the use of an appropriate
subtraction hybridization protocol, which can efficiently
5   reduce cDNA complexity and generate stable populations of
cDNAs for analysis.

Previous studies demonstrate that different gene cloning
strategies, including DDRT-PCR, subtraction hybridization
10 and electronic display, identify distinct subsets of
differentially expressed genes (18). These results
suggest that a single approach for gene identification
may not identify the complete spectrum of differentially
expressed genes. Similarly, RSDD and DDRT-PCR do not
15 resolve the same differentially expressed bands (Fig. 1).
Unique bands identified in DDRT-PCR that were
differentially expressed when analyzed by Northern
blotting were not the same as those found using RSDD and
vise versa (data not shown). These results are not
20 surprising, since, as indicated above, subtraction
hybridization and differential RNA display identified
distinct differentially expressed genes (18). Apparently,
specific differentially expressed genes are lost during
subtraction hybridization and differential RNA display of
25 subtracted cDNAs. On the basis of these considerations,
it will be essential to use multiple gene discovery
approaches to identify and clone the complete spectrum of
differentially expressed genes.

30 DDRT-PCR can generate large numbers of differentially
displayed bands making subsequent analysis both labor
intensive and a daunting challenge. In order to reduce
these limitations of DDRT-PCR, RSDD has been used in
combination with reverse Northern analyses of isolated
35 cDNAs. Gel extracted cDNA fragments were reamplified,
dot-blotted on Nylon membranes and successively probed
with reverse transcribed ^{32}P-cDNA from E11 or E11-NMT RNAs

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(Fig. 2). Signals were detected in 181 reamplified bands out of 234 (77%).

The signal intensities of the various cDNAs in reverse
5   Northern analysis were quantified and normalized against
that of GAPDH, which remained unchanged in E11 and
E11-NMT cells. Progression elevated gene-3 (PEG-3)(6) was
used as an additional control, to verify increased
expression in E11-NMT versus E11 cells. In the reverse
10   Northern analyses, PEG-3 levels were 4-fold higher in
E11-NMT than in E11 cells, which coincided with Northern
blotting results, thereby demonstrating the concordance
of reverse Northern and Northern assays. A $\geq$ 1.8-fold
differential cut-off (after normalization for GAPDH
15   expression) was used to identify and isolate cDNA bands
displaying modified expression in E11 versus E11-NMT
cells. This resulted in the identification of 7 cDNAs
with higher expression in E11 versus E11-NMT cells and 65
cDNAs with elevated expression in E11-NMT versus E11
20   cells. These results suggest that tumor progression in
E11-NMT cells correlates with increased expression of a
large number of genes, whereas only a smaller subset of
genes display decreased expression.

25   A problem frequently encountered in DDRT-PCR, that is
reduced but still can occur in RSDD, is the isolation of
multiple cDNA species from what appears to be a single
amplified band. When this occurs, these multiple species
can produce spurious results when analyzed by reverse
30   Northern analyses. For example, if two distinct species
are isolated, one displaying modified expression and a
second not displaying modified expression, an accurate
estimate of differential expression will not be obtained
by reverse Northern analysis. In this case, a number of
35   potential false positives generated using reverse
Northern analyses, may in reality not be false positives,
but instead may represent multiple cDNAs. By performing

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single strand conformational polymorphism (SSCP) or reverse Northern analyses using cloned cDNA populations (33,34) this problem can be ameliorated.

5   The expression pattern of representative RSDD-derived cDNAs in E11 versus E11-NMT and in a more expanded E11/E11-NMT progression cell culture series is shown in Figs. 29 and 30, respectively. Reverse Northern results correlated well with Northern blots using E11 and E11-NMT
10   (~75% concordance) or a larger panel of cells differentially displaying the progression phenotype, including progression negative E11, CREF x E11-NMT F1 and F2, E11 x E11-NMT A6, E11 x E11-NMT 3b, E11-NMT Aza B1 and Aza C1 cells, and progression positive E11-NMT, CREF
15   x E11-NMT R1 and R2, E11 x E11-NMT A6TD, E11 x E11-NMT IIa, E11-Ras R12 and E11-HPV E6/E7 cells. Sequence analysis of the various PEGen cDNAs identified both unknown and known genes (Table 3). Five of 10 PEGen cDNAs (50%) were classified as novel sequences since no matches
20   were found in current DNA databases. Novel PEGen cDNAs include, PEGen 13, 14, 24, 28 and 32. Known PEGen genes included PEGen 7 (human papilloma virus-16 early region 1 binding protein; HPV16 E1BP), PEGen 8 (phosphofructokinase kinase C; PFK-C), PEGen 21 (a
25   fibroblast growth factor-4 inducible gene; FIN 14), PEGen 26 (poly ADP-ribose polymerase) and PEGen 30 (rat esp1 homology). In the case of the PSGen cDNAs, six of six (100%) were novel, including PSGen 12, 13, 26, 27, 28 and 29 (Table 3).

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Table 3. PEGen and PSGen genes isolated using RSDD

Nomenclature[a]	Identity[b]	Homology (%)[c]
PEGen 7	Human HPV16 E1BP	90
PEGen 8	Rat phospho-	
	fructokinase C (PFK-C)	100
PEGen 13	Unknown	Novel
PEGen 14	Unknown	Novel
PEGen 21	Murine FIN 14	94
PEGen 24	Unknown	Novel
PEGen 26	Rat poly ADP-ribose	
	polymerase 100	
PEGen 28	Unknown	Novel
PEGen 30	Rat esp1	98
PEGen 32	Novel	Novel
PSGen 12	Unknown	Novel
PSGen 13	Unknown	Novel
PSGen 26	Unknown	Novel
PSGen 27	Unknown	Novel
PSGen 28	Unknown	Novel
PSGen 29	Unknown	Novel

[a]PEGen are progression elevated genes that display elevated expression in E11-NMT versus E11 cells. PSGen are progression suppressed genes that display elevated expression in E11 versus E11-NMT cells.

[b]Sequences have compared with reported genes in various DNA data bases (including GenBank and EMBL) and identification with known genes are indicated. Genes without homology to currently reported genes are indicated as unknown.

[c]percentage homology with known sequences, either human, rat or mouse is indicated.

Where no homology exsists the cDNA is considered novel.

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PEGen 7 is expressed at ~ 4-fold higher levels in E11-NMT than in E11 cells. PEGen 7 is ~98% homologous to 16E1-BP, a cDNA encoding a protein identified using the yeast two-hybrid assay that interacts with human papillomavirus type 16 E1 protein (35). 16E1-BP encodes a 432aa protein of unknown function but does contain an ATPase signature motif (Gly-X4-Gly consensus ATP binding motif at aa 179 through 186). 16E1-BP appears to be a form of TRIP13, a protein previously shown to bind thyroid hormone receptor in yeast two-hybrid assays. The role of PEGen 7/16E1-BP in the progression phenotype in the E11/E11-NMT progression model is not known. Additional studies are necessary to determine if this gene change is associative or causative of transformation progression.

PEGen 8 is expressed at ~3- to 4- fold higher levels in E11-NMT than in E11 cells. PEGen 8 shows 100% homology to rat phosphofructokinase C (PFK-C)(36). PFK catalyzes the rate-limiting and committed step in glycolysis, the conversion of fructose 6-phosphate to fructose 1,6-biphosphate. Three subunit isozymes of PFK have been identified, that form homo- and heterotetramers with differing catalytic and allosteric properties. PFK-M is specific for cardiac and skeletal muscle, PFK-L is expressed in many tissues but is most abundant in the liver and PFK-C is expressed in several brain regions and the anterior pituitary but not in liver, skeletal muscle, or several other human tissues. The cDNA of PFK-C isolated from a rat hypothalamic cDNA library is 2643 bp and encodes a protein of 765aa (~36). In a recent study Sanchez-Martinez and Aragon (37), demonstrated that PFK-C is the predominant form of PFK in ascites tumor cells (obtained from a transplantable mouse carcinoma of mammary origin), whereas PFK-L is most abundant in the normal mammary gland. These results suggest the interesting possibility that PFK-C might contribute to the malignant nature of specific target cells. The role

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presently reported of PEGen 8/PFK-C in progression in the
E11/E11-NMT model remains to be determined.

PEGen 21 is expressed at ~3- to 4-fold higher levels in
5   E11-NMT than in E11 cells. PEGen 21 displays ~98%
homology with the fibroblast growth factor-4 inducible
gene FIN-14 (38). FIN-14 is a novel cDNA of unknown
function that hybridizes with a 4.5 kb mRNA that is
induced 4-fold in NIH 3T3 mouse cells following treatment
10   with FGF-4. The induction of FIN-14 occurs late (18 hr)
after treatment with FGF-4 and does not occur when cells
are treated for 18 hr with FGF-4 in the presence of
cycloheximide (38). These results confirm that FIN-14
encodes a late-inducible gene. Moreover, nuclear run-on
15   assays document that FIN-14 is transcriptionally
activated in NIH 3T3 cells following growth factor
stimulation. Tissue distribution studies indicate
expression of a single mRNA species in the kidney with
low levels of expression observed in several other
20   tissues including testis and thymus. Mouse embryogenesis
studies indicate that FIN-14 expression occurs
constitutively in mouse embryos between day 10.5 and
15.5. Unlike NIH 3T3, FIN-14 was constitutively expressed
in PC12 cells and its level did not vary appreciably in
25   response to growth factor stimulation. The role of PEGen
21/FIN-14 in progression in E11/E11-NMT model system is
not currently known.

PEGen 26 is expressed at ~3- to 4-fold higher levels in
30   E11-NMT than in E11 cells. This cDNA is identical to rat
poly(ADP-ribose) polymerase (PARP)(39). PARP contributes
to the ability of eukaryotic cells to contend with both
environmental and endogenous genotoxic agents (40). PARP
is a nuclear enzyme that binds to DNA breaks and then
35   catalyzes the covalent modification of acceptor proteins
with poly(ADP-ribose) (39,40). PARP activity contributes
to the recovery of proliferating cells from DNA damage

and to the maintenance of genomic stability, which may be regulated by effects on chromatin structure, DNA base-excision repair and cell cycle regulation (39,40). The role of PEGen 26/PARP in mediating the progression phenotype is not currently known. However, since cancer is a progressive disease characterized by the accumulation of genetic alterations in the evolving tumor (6), it is tempting to speculate that overexpression of PEGen 26/PARP in E11-NMT may facilitate the ability of these aggressive cancer cells to maintain genomic stability during cancer progression. In this context, PEGen 26/PARP may be an integral component of progression. This hypothesis is readily testable. PEGen 30 is expressed at 2- to 3-fold higher levels in E11-NMT than in E11 cells. This cDNA displays ~98.5% homology to rat esp1 (41). Rat esp1 encodes a 24-kDa nuclear protein which is the rat homologue of Drosophila Enhancer of split., a gene involved in ventral ectodermal development in Drosophila (41). PEGen 30 appears to be a homologue of esp1, since the message detected in E11 and E11-NMT cells (~4 kb) is larger in size than the reported esp1 transcript (1.3 kb)(41). The role of PEGen 30/esp1 in tumor progression in E11/E11-NMT model system remains to be determined.

The PSGen cDNAs, 12, 13, 26, 27, 28 and 29, consist of sequences without homology to those in various DNA data bases. Expression of PSGen 12 and PSGen 13 cDNAs is ~3- to 4-fold higher in E11 versus E11-NMT cells (Fig. 29). It is not currently known whether these genes simply correlate with or functionally regulate the progression phenotype. The identification of full-length cDNAs for PSGen-12 and PSGen-13, as well as the other novel PSGen and PEGen cDNAs, are in progress and once isolated experiments can be conducted to directly define the role of these progression-related genes in cancer progression.

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Presently demonstrated is a modified gene-identification
and gene-cloning technique, RSDD, that can efficiently
identify differentially expressed cDNAs. As predicted,
subtractive hybridization prior to differential RNA
display greatly reduces band complexity, a problem
encountered in standard DDRT-PCR in which RNA samples are
directly analyzed without subtraction. Unlike a previous
report using subtracted cDNAs processed through
successive rounds of PCR (25,42), common bands were
eliminated using reciprocally subtracted cDNA libraries
that had not been processed using PCR. In addition to
subtraction   hybridization,   the   discovery   of
differentially expressed genes was further streamlined by
using reverse Northern analyses with isolated cDNAs. With
3 single anchored oligo dT primers and 18 arbitrary 5'
primers, 72 bands were identified that displayed
differential expression using reverse Northern analysis.
Currently, 38 cDNA species have been analyzed by Northern
blotting and 31 (~82%) displayed differential expression
in E11 versus E11-NMT cells. Sequence analysis of the
cloned cDNA fragments revealed 16 different genes,
including 11 novel genes not reported in recent DNA
databases. RSDD represents a method of choice either as
a more efficient and less time consuming modification of
the differential RNA display strategy or as a screening
methodology for identifying differentially expressed
genes in reciprocally subtracted cDNA libraries.
Moreover, the ability of RSDD to identify differentially
expressed genes that are dissimilar to those recognized
using standard DDRT-PCR or subtraction hybridization
indicates that this approach will be a valuable adjunct
in cloning the complete repertoire of differentially
expressed gene changes occurring between complex genomes.

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What is claimed is:

1.   A method for identifying differentially expressed
        nucleic acids between two samples, comprising:
5   a.   selecting a first and second nucleic acid
                        sample, wherein the nucleic acid samples
                        contain a repertoire of nucleic acids;
                b.   performing reciprocal subtraction between
                        the nucleic acid samples to produce two
10   subtracted nucleic acid samples;
                c.   amplifying the two subtracted nucleic
                        acid samples; and
                d.   comparing the two subtracted nucleic acid
                        samples to identify differentially
15   expressed nucleic acids.

2.   A method for identifying differentially expressed
        nucleic acids between two samples, comprising:
                a.   selecting a first and second nucleic acid
20   sample, wherein the nucleic acid samples
                        contain a repertoire of nucleic acids;
                b.   amplifying the two nucleic acid samples;
                c.   performing reciprocal subtraction between
                        the amplified nucleic acid samples to
25   produce two subtracted nucleic acid
                        samples; and
                d.   comparing the two subtracted nucleic acid
                        samples to identify differentially
                        expressed nucleic acids.

30
3.   The method of claim 2, wherein the two subtracted
        nucleic acid samples from step c are amplified prior
        to the comparing of step d.

35 4. The method of claim 1 or 2, wherein the each of the
        nucleic acid samples comprises a library of nucleic
        acids.

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5.   The method of claim 1 or 2, wherein the nucleic acid
       samples are mRNA or cDNA derived from mRNA.

6.   The method of claim 1 or 2, wherein the nucleic acid
       samples are obtained from total RNA from E11 and
       E11-NMT cells.

7.   The method of claim 1 or 2, wherein the first and
       second nucleic acid samples are obtained from cells
       that differ in their exposure to external factors or
       in their gene expression.

8.   The method of claim 1 or 2, wherein the first and
       second nucleic acid samples are obtained from cells
       in different developmental stages.

9.   The method of claim 1 or 2, wherein the amplifying
       of step (d) comprises PCR amplification.

10.   The method of claim 9, wherein the PCR amplification
       uses a set of random primers.

11.   The method of claim 9, wherein the 3' primer used in
       the PCR amplification is a single anchor oligo dT 3'
       primer.

12.   The method of claim 9, wherein the 5' primer is an
       arbitrary primer.

13.   The method of claim 1 or 2, wherein the comparing of
       step (e) comprises using a gel to separate the
       nucleic acids from both of the libraries.

14.   The method of claim 1 or 2, further comprising PCR
       amplifying the first and second nucleic acid
       samples.

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15. The method of claim 1 or 2, further comprising
     reamplifying differentially expressed nucleic acids.

16. The method of claim 1 or 2, wherein the comparing of
     step (e) comprises comparing the quantities of the
     two amplified differentially expressed nucleic
     acids.

17. The method of claim 1 or 2, wherein differences in
     the quantities of nucleic acid between the two
     subtracted libraries are electronically quantified.

18. The method of claim 1 or 2, wherein the libraries of
     step (b) are constructed with λ-ZAP cDNA library
     kits.

19. The isolated nucleic acid identified by the method
     of claim 1 or 2, wherein the nucleic acid was not
     previously known.

20. The isolated nucleic acid of claim 19, wherein the
     isolated nucleic acid is the nucleic acid designated
     PSGen 12.

21. The isolated nucleic acid of claim 19, wherein the
     isolated nucleic acid is the nucleic acid designated
     PSGen 13.

22. The isolated nucleic acid of claim 19, wherein the
     isolated nucleic acid is the nucleic acid designated
     PSGen 23.

23. The isolated nucleic acid of claim 19, wherein the
     isolated nucleic acid is the nucleic acid designated
     PSGen 24.

24. The isolated nucleic acid of claim 19, wherein the

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isolated nucleic acid is the nucleic acid designated
PSGen 25.

25.   The isolated nucleic acid of claim 19, wherein the
isolated nucleic acid is the nucleic acid designated
PSGen 26.

26.   The isolated nucleic acid of claim 19, wherein the
isolated nucleic acid is the nucleic acid designated
PSGen 27.

27.   The isolated nucleic acid of claim 19, wherein the
isolated nucleic acid is the nucleic acid designated
PSGen 28.

28.   The isolated nucleic acid of claim 19, wherein the
isolated nucleic acid is the nucleic acid designated
PSGen 29.

29.   The isolated nucleic acid of claim 19, wherein the
isolated nucleic acid is the nucleic acid designated
PEGen 13.

30.   The isolated nucleic acid of claim 19, wherein the
isolated nucleic acid is the nucleic acid designated
PEGen 14.

31.   The isolated nucleic acid of claim 19, wherein the
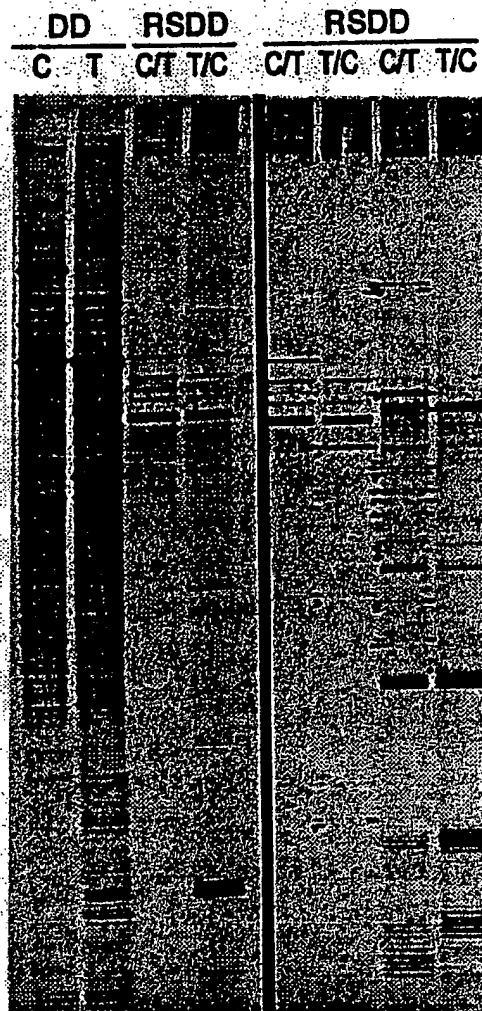isolated nucleic acid is the nucleic acid designated
PEGen 15.

32.   The isolated nucleic acid of claim 19, wherein the
isolated nucleic acid is the nucleic acid designated
PEGen 24.

33.   The isolated nucleic acid of claim 19, wherein the
isolated nucleic acid is the nucleic acid designated

PEGen 28.

34.   The isolated nucleic acid of claim 19, wherein the
       isolated nucleic acid is the nucleic acid designated
5   PEGen 32.

35.   The isolated nucleic acid of claim 19, wherein the
       isolated nucleic acid is the nucleic acid designated
       PEGen 42.

10

36.   The isolated nucleic acid of claim 19, wherein the
       isolated nucleic acid is the nucleic acid designated
       PEGen 43.

15   37.   The isolated nucleic acid of claim 19, wherein the
       isolated nucleic acid is the nucleic acid designated
       PEGen 44.

38.   The isolated nucleic acid of claim 19, wherein the
20   isolated nucleic acid is the nucleic acid designated
       PEGen 48.

39.   The isolated nucleic acid molecule of claim 19 which
       comprises:
25   (a)   one of the nucleic acid sequences as set forth
             in Figure 35;

       (b)   a sequence being degenerated to a sequence of
             (a) as a result of the genetic code;

30

       (c)   a sequence encoding one of the amino acid
             sequences as set forth in Figure 35.

       (d)   a sequence of at least 12 nucleotides capable
35   of specifically hybridizing to the sequence of
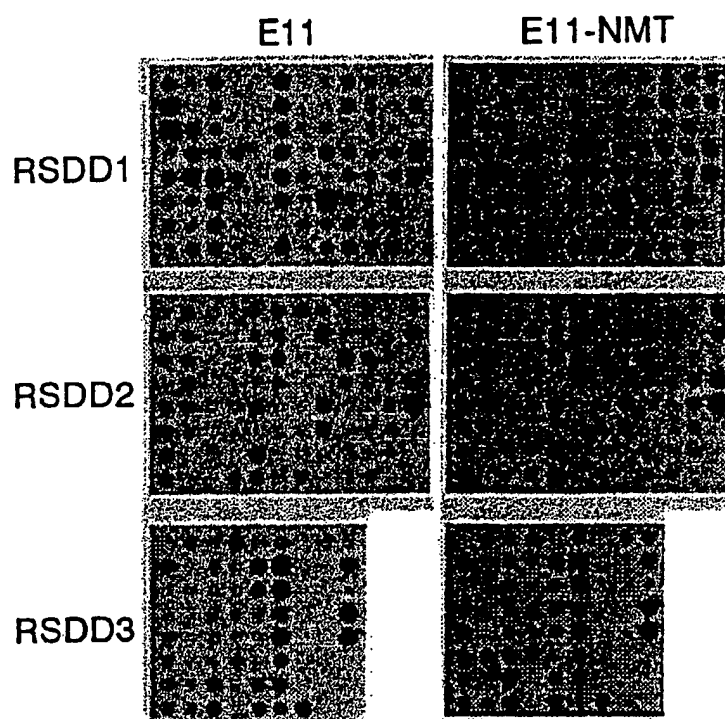   (a), (b) or (c)

40.   A purified polypeptide comprising one of the amino
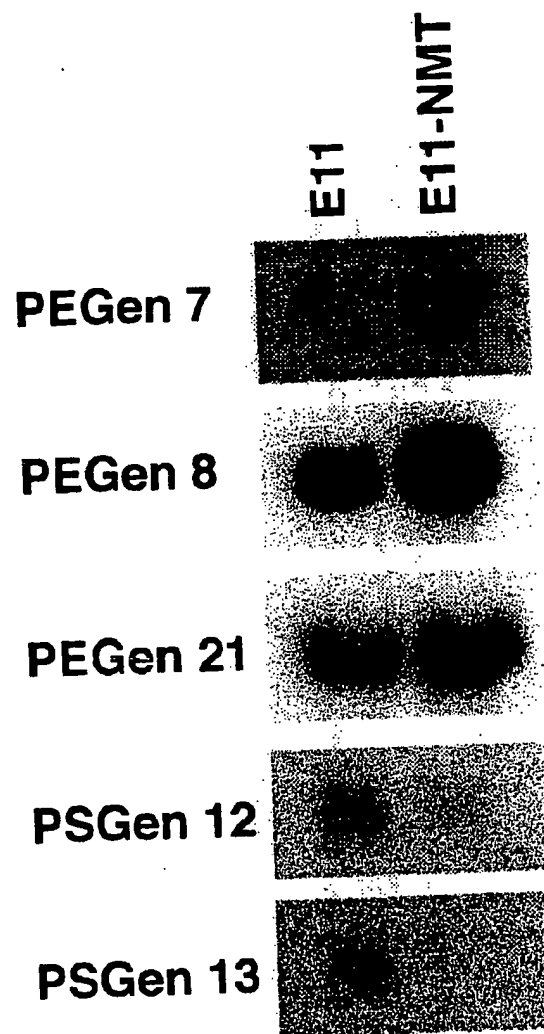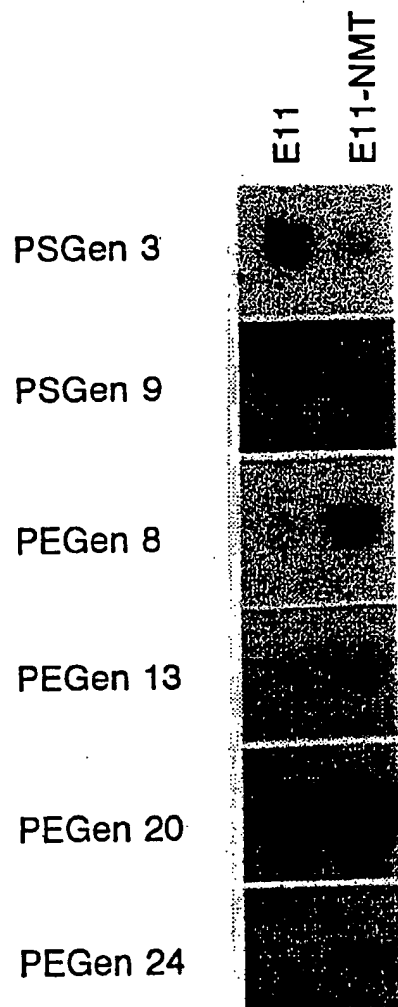   acid sequence as set forth in Figure 35.

FIG. 1

FIG. 2

FIG. 3A

FIG. 3B

FIG. 4

Columns (left to right):
E11 (-), E11-NMT (+), CREFxE11-NMT F1 (-), CREFxE11-NMT F2 (-), CREFxE11-NMT R1(+), CREFxE11-NMT R2 (+), E11xE11-NMT A6 (-), E11xE11-NMT A6TD (+), E11xE11-NMT 3b (-), E11xE11-NMT IIa (+), E11-NMT Aza B1 (-), E11-NMT Aza C1 (-), E11-Ras R12 (+), E11-HPV E6/E7 (+)

Rows: PEGen 7, PEGen 8, PSGen 12, PSGen 13

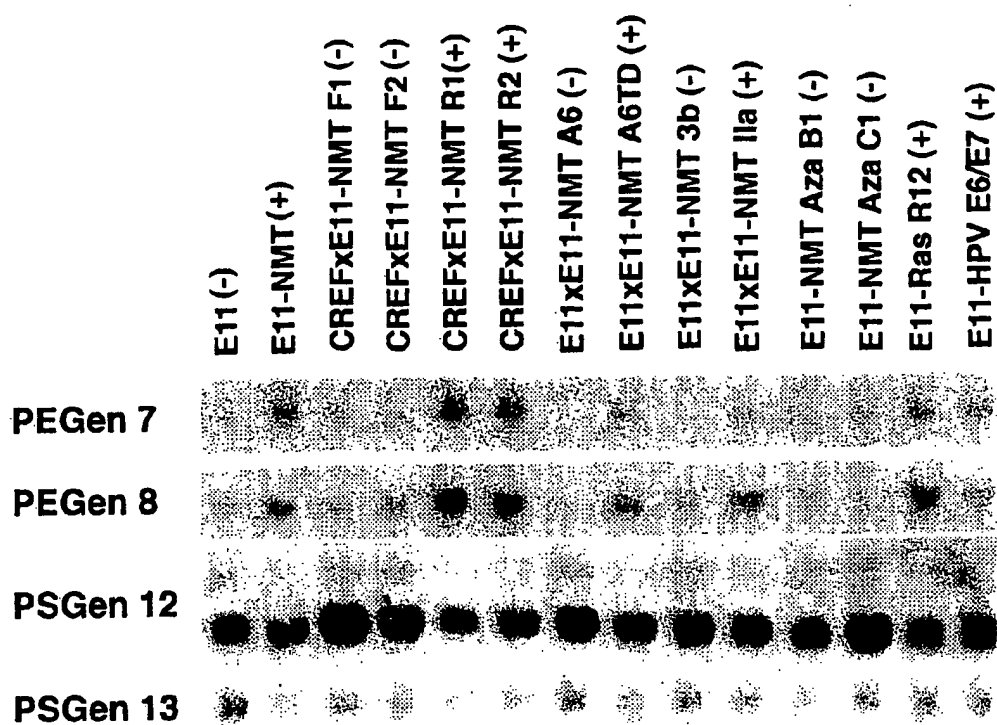**6/23**

FIG. 5

PEGen 7-90% homology to human HPV16 E1BP

```
TAAANCGGTG GTACTGCTGC ACGGTCCTCC GGGTACTGGA AAGACATCCC
TTTGTAAGGC ATTAGCCCAG AAACTGACCA TCAGACTGTC AANCAGGTAC
CGGTATGGCC AGTTAATTGA AATAAACAGC CACAGCCTAT TTTCTAAGTG
GTNTTCAGAA AGTGGCAAGT TGGTAACTAA GATGTTCCAG AAGATTCANG
ACTTGATTGA TGATAANNAA NCTTTGGTGT TTGTCCTGAT TGATGANGTA
AGCACTCANN GGTACTCATT CTTNGTCTGC ATTGCCTCTT GCTATTACTG
CCTGATCCCT CTCATTTGGT TCACTGTGTC GCNANCTCTT TTCTATGGAT
CTTTTCCNAN CCACCCGTTT C
```

FIG. 6

PEGen 8-Rat phosphofructose kinase C

```
GTGACGTAGG GTCTGTTGCG TCAATGGTTA TAGCAAGTGA TGCTCTCTGA
TTATTACTGC TGACAATACT CGGCCAACAA TTCTTGCATA GAGTGCTGAT
AAATAACTAT GTTACAAAAA GGGGTGGTCC CTGGAGAACA TTACAGGCTT
CCCTAGGTAA GTGTGCAGGT CAGGAGACGG CATATTCAAT CAGATGGCTG
ATAGTTCTCC GTGGTTATGC ACCGGCTCCA GCTTGCCTAC GTCAC
```

FIG. 7

PEGen 13-Novel

```
GCAGCATGAT GAATTTAATG CAACAGTCAT AGCAGGGCAA GGGGAGAGAA
AGGCAGATGG ACTATCTGCA TCATCAAGCG AGGGCTTGTG TCGGCGGCTA
TGTGCAGAGA CGAGCAGGGC GAGGCACTTA AAAGCTGCTN GATGAAAATC
CACCCAGGAG AANTCTGGGC CTACGTCA
```

```
TGACGTAGGC CCAGACTTCT CCTGGGTGGA TTTTCATCCA GCAGCTTTTA
AGTGCCTCGC CCTGCTCGTC TCTGCACATA GCCGCCGACA CAAGCCCTCG
CTTGATGATG CAGATAGTCC ATCTGCCTTT CTCTCCCCTT GCCCTGCTAT
GACTGTTGCA TTAAATTCAT CATGCTGCCA AAAAAAAAAA A
```

FIG. 8

PEGen 14-Novel

```
GCCATAAATA CACTTTATTT CATTCGAAAT GCATAATCAC ACTGGGAGCA
CTCCCTTTGG AGCACTCCTC TAGCAGCAGG TCCGAAGTGC TCCAGCATCG
TCAGCTGGCT CCAACACCTA CGTC
```

FIG. 9

PEGen 15-Novel

```
TTTTTTTTTT TTTGGAAACA GAATAAAGTG CTTTATTCTC TGGCTGGCTC
TCCTACGTCA C
```

FIG. 10
PEGen 21-94% homology to mouse FIN 14

```
TCGGCGATAG CATTGGAGCA AGTCTTATCA GCAAGCAATG TTTTCAGTTA
TGTTTCAAAG TTAAGAATGG GTTTAAACTT GCTGAACGTA AAGATTGACC
CTCAAGTCAC TGTAGCTTTA GTACTTGCTT ATTGTATTAG TTTANATGCT
AGCACCGCAT GTGCTCTGCA TATTCTGGTT TTATTAAAAT AAAAAGTTGA
ACTGCAAAAA AAAAAA
```

FIG. 11
PEGen 24-Novel

```
TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TNGCCAGGCT
ATGTCTCAGA CTTTATTATT ATTATTATTA TTATTATTAT TATAAATAAA
ACATGTNCTT TCAATTAGGT TACAANAGTA TTTATCTCCA TAACGCTTCT
TCATACATCC TTAGTTTTGG ATTAAAGTAC CATCCACCCC AACTCAAACT
GTAACCCCCA GTAATCCCCT CTAACGTGGA AATTTCTGGT TTAACAACTC
AGTTAACTGC CCCACAAACA GTGGGAGGCC GCTCTTGCAT GGCTATGCCA
CGTAACCCTT CACTGCTTCA CTTCTTCGCT GGCT
```

FIG. 12
PEGen 26-Rat poly ADP-ribose polymerase.

```
GACCGCTTGT ACCATCCAAC TTGCTTTGTC TTCTGCAGAG AGGAGGCTAA
AGCCCTTGAG CTGGCTGGCA CTGTACTCAG GCCGGAAGCC CAGCTCGTCC
CGGTTCTTGA CAAAGCAAGT TGGATGGTAC AAGCGG
```

FIG. 13
PEGen 28-Novel

```
TGCCGAGCTG GGTATTGTGA CGGTTGATAA TGGCGGCATC ATGTTGCCAG
GTACCGGGTA AGCAGACCTC AGAGCACAGC TTATTGTCCA GTGCTTTCAC
GCTCGCGACG TCAAAGTCAT TGTTATTGTC ACACTCCATG CCTAGAAATG
CGCATGTCCT CTGGCCATCT TCTTGCACAG GGGATCTGTC CTCTTCCTCC
ATGATATCAT TTCCCTCTGC ATCCTGCTCT CCAGCTGGAA GGCCAGCAAA
ATTGCTGTCT GGGGACTCTG CTGGGGTCTC CTCCTCTTCT GAAGGGGCCC
TGCTAGCAGC TCGGCA
```

FIG. 14
PEGen 42-Novel

```
AGGGGTCTTG ATGGACTTGG GTCGGACATC TTAGTGACCT GTGAATTCTT
CTGTGGAGGC TGAGTCTCAC GTAGCCGAGT TTAATATCTG TGCTATTTAC
TAAAGTATCT GCCACCAAAT TGTACCAACT CATAGTTTTA TATGAATGTT
GATGAGTCTG TATCATAAAT AGAATTGTTG ATACATCCTT AATTTGTGCA
ATATTGTATG AAGAAGATTG TTATCAATTA AAACCACGCC TCTTTATGAT
CCTNNNAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA
AACCNCCTCA AATCCATNGG TTCTAACCCA AAACCCT
```

FIG. 15
PEGen 43-Novel

```
TTTTTTTTTT CATACACCAT CAAACCAATT TTATTTCTAT AGCAACGTTT
CTCACGTCTG AACCTGAGAA TAAGTCACCA GCTCTTGACA GTAAACATGG
GCCCTATCAA ATTATATTAG ACTCCTCAGT GTCCCGCCAT GTGGCCTTGC
ACCAAATCAA TTAGTTTGAG GGCCAAAATC CTGTTGGGTT TCAAATAAAG
TGTCAGGTCA TAAGGAGGGG GAGGGACTCA ATTCATGGGA ACATTTTTAC
CTGTTCAAAT AGATAAACTG AATTGCCCTA TCTGTGGTCA CCTGGATCCA
AGACCCT
```

FIG. 16
PEGen 44-Novel

```
CCCTGACGAT AAATGGTAAG GAACTTTTTT TTTTTTTTTT TTTTTTTTTT
TTTTTTTTNC GAAATAAACA AACACAGCTT ATTATTTGGG GGAACATTAA
NTTCTATAAN TGAACACAAA ANAAAATTAA NANTTAATGG GGGGGTANAA
GGGACTTTGA ATCTATCTGG TATCATGACA TTGAAGCANA NACCTGANTG
ACCAGAAAGA GAGAGAGAGA GAGAGAGAGA GAGAGAGAGA GAGAGGTTTC
ATATGAGCTA GTGTTACAGG CTTTATTAGT CTATTAGTCA GGGACC
```

FIG. 17
PEGen 48-Novel

```
AATCGGGCTG GATGGGTGTA TCCGGCACTG TTTCGTAGCG GCAGCAACTG
GGTGCTTCTA TCTGAAAGCG GGCTTCACAA AAACTACTGC GCCACCCGAC
TCGCTGCGGC ATCGCCCGGT GGCGAGTACC GTATCGCCTT TCCTGGTGCA
GAAGAAGTGT TTACAGGAGG CGGTCATTTA CCGCAATCTG ATTCTGTTTT
TTATTCTCCC TGGCGGGTGA TCGCGATCGG CAGTTTGAAA ACGATCGTTG
AATCCACGCT CGGGAATGAT GTGGCTTCGC CGCCAACGCT TACTGACATT
TCATTTGTAC AGCCCGATT
```

FIG. 18
PSGen 1-80% homology to *B. taurus* supervillin

```
GCCGAGCTGT GTAAAACCAT CTATCCTCTG GCAGATCTAC TTGCCAGGCC
ACTCCCAGGG GGGGTAGACC CTCTAAAGCT TGAGATTTAT CTTACAGATG
AAGACTTCGA GTTTGCACTC GACATGACCA GAGATGAATT CAACGCACTG
CCCACCTGGA AGCAAATGAA CCTGAAGAAA GCGAAAGGCC TGTTCTGAGG
GTGAGATGAC AGCCACAGAG AGGTCACTGC CACTAGACCA GAAAGTGGAT
GGAGATATAT ATTTGGACTG GTGTTTTTTT CTGTCAG
```

FIG. 19

PSGen 2-91% homology to human HTLV-1 Tax interacting protein

```
ATCGGGCTGC AGATTGGAGA CAAGATCATG CAGGTGAACG GCTGGGACAT
GACCATGGTC ACTCATGACC AGGCTCGGAA GCGGCTCACC AAACGTTCGG
AGGAAGTGGT CCGCCTGCTG GTGACTCGGC AGTCTCTGCA GAAGGCCGTA
CAGCAGTCCA TGCTGTCATA GCTGTAGTCA GCCTAGACTT CTGCCCACTG
ACCTTTTNGG GCACTGAGAA CACATCCACG CTCTGTCTGT ATCTAGTTCT
GGCTTCTGCT GTGTGCTANG CCCCAGCTCT GAGGAGTAAC AGCTGATCCC
AAAGGTCCAA GCCAACCTTC TTACCCCTCA GCCCCCANCC CGAT
```

FIG. 20

PSGen 4-Rat proteasome activator

```
TTTTTTTTTT TTTGGGCAAC TATGTATTTA TTGTGTTTGG AAGGCAGAGT
GAGGGAGGAG ACCCCAGCAG GAAGAAGACT GGGTGCAGTC TAGAGTTCCT
AGTCAAGAGT AGGAAGGTTT CTGTTATACC CATCATAGAA CGAGAGAGGG
GGCTCAATAG ATCATCCCCT TTGTCTCTCC ACGGGGCTTC TTGAGCTTCT
CAAAGTTCTT CAGGATGATG TCATATAACA CAGCATAAGC GTTACGGATC
TCCATGACCA TCAGCCGGAT CTCCTGGTAT TCCGCCTCGT CCAGCTCGGC
```

FIG. 21

PSGen 10-Rat Ferritin Heavy Chain

```
AANATCTGCT TAAAAGTTCT TTAATTTGTA CCATTTCTTC AAATAAAGAA
TTTTGGTACA AATTAAAGAA CTTTTAAGCA GATGTTTTGG TGCAACTAAT
AGAAAAGATA AAGGCAGCCT GACATGCATG CACTGCCTCA GTGACCAGTA
AAGTCACATG NCCTTGGGAC GTCAGCTTAG NTTTATCACN GTGTCCCAGG
GGTGCTTGTC AAAGAGATAT TCTGCCATGC CAGATTCAGG GGCTCCCATC
TTGCGTAAGT TGGTCACGTG GTCACCCAGT TCTTTAATGG ATTTCACCTG
CTCATTCAGG TAATGCGTCT CAATGAAGTC ACATAAGTGG GGATCATTCT
TGTCAGTAGC CAGTTTGTGA AGTTCCAGTA GTGACTGATT CACACTCTTT
TCCAAGTGCA GTGCACACTC CATTGCATTC AGCCCGCTCT CCCAGTCATC
ACGGTCACNT A
```

FIG. 22

PSGen 12-Novel

```
TGACGTAGGG CCGAGAGCAA CAAGCACAGA ACTCCTTCTC CAGTTTCACC
CTGATGAAGT TGAGGCACTC TTCTGCACTG GGAGGGGCCA GCCTGGGGGC
CAGGCACATT GGACACCACC TTCCCATGGA CTACAGCGTC AATGCCATTG
CCTTCTATTC CTATACCTTC TAGGGGCTGC CCCTCTTCCC ATTCAGCCAA
CACTGAGTGT TGGGAGATTT CTCTTTTTTA AAAACACATG AGAAAATAAA
TGCACTTTAC TCCCTCCCCA AAAAAAAAAA
```

FIG. 23

PSGen 13-Novel

```
GTAGGCAATA AAATGTTTTC AGAGGTGCGA AAAAGCTTTT GTTTTCTTAA
ACCATTCTTA GTCTCTGCCA CACTTGACAC TCCGTCAAAG TGAGAAGCGA
ACTAAAGACC AACTGCGGTG GAAAATATTA TGTTTATGTA ATAAAAAAAA
ATCATGTAAC TGCAAAAAAA AAAAAAA
```

FIG. 24

PSGen 23-Novel

```
TGCCGAGCTG AAAACATACA TCCGCACCGG GTTGAGATAG CTGGCCCTCC
GTCCCCGGGC ATACTCTTTG GATAAGAACC CCGGCCTTGT TACCAGGTAC
CGGAGTGAGC TGAAAAATTT ACCGTCGAAA TGGGTGATGT CCTGGAAAAA
ATGGTTCACC AGCTGCCAGG CAGATTCTTT GGGTTCCACA TTTTCCTGCC
CACAGATGTG GCAGAAGCGG TCAAGTAATG CAGCATTACA ATTGAGGCAG
ATCTTTTCTT TTCTTTCCTT GGAGTGGCTC AACCAGCGAT TTTGGTTAAA
AATAATCAAA AAAGCGACGG CAAAACTTTT GTTATATTCC CGCCTGTGGC
ATTTGAACTG TGCCCGGCAA CCGAATAACT TTTAATTTTG AAAATAAAAT
GCATACTAGA TTTTTAGCGG TTGCCTCCTG GCCATTGCTT CAGGCGCCNG
CACAGCGTCA GCCCAGTTTT ACCACNANGA ATATCCTAAG CGTTGAAACA
GGGCACAGCC GAAAAAAACN CTGGCNACAA AAAANATCCG GACATCCTTT
TTCCAATTTT GAAACCGAAN GCNCGCAAAC NAAGGTTCTT CGGGAAAAAA
AATCGCCAAA ATACNCGANA TCAAACTNTC CAA
```

FIG. 25

PSGen 24-Novel

```
TGCCGAGCTG GGGGGAGTTC CAGGAATTTG TGGACTATTT CCAGGAGGAA
TTGAGGAATC TAGAAGTAAT AAGAACTTCA CAAGTAGAAC AACAGAGTTA
ATTGACCTCT ATCCTTAAGA GTTACCAGAG AATTATTAAA AAACTAAAGA
ACAATCAAAG CCTGGTCCTG TGCCACCACC CAAAAACATG TATAGCCTAT
GTGCAGCTCG GCA
```

FIG. 26

PSGen 25-Novel

```
CTCANAGGGC NNNTTNGNGG NCNTCATGCN CCAGGNTCCN NCCCCCANAN
GANCNNCCNG GTAAACTACA CNGGAGTACT TAAGTGGACA NNCCACATGC
GANGGNCAAG GGGATCACCN TCNCTCCTNC AGNCTNTNCG TGNCTCTCCT
GTNCNTNCAC TGCCNCANAA NGGANGCNCN NNCTCCTATC TGTNTACAGN
AAACNTNGCN CTNNCTCTAA GCTCNCCCAC TNTGTGGAAA GGCNATGTGT
GCGTGCCTCT CCCCTATCAC GGCNGTTTGC NAAANGGGGA TGTNCTGCNC
GGCGATGAAG TTNGGTCACT CCATGTTTCC CAGTCCNACC TGTTAGACNA
AGNATTGNAN TGTGATACGA CTCNCTGTAA GGGGANTNGC GGACCCAGTA
TGTTTGGCCC NACNNCCACT TCTTTAAATG GTGGCTAACG GCGCTTCCTA
GNATAAACAC TATTGGTCCC CCCCTCTGCA GNACCCNTTA CTTCCGNANA
AAAATTGTTG TCNTGATCCG CGACAACCAC ACCGTCTGTN GNTTTTAGTT
GCAACNCNNA TCNCTCCAAA AAAGTTTCAG AAATCTTCAT TTTCCCNGGT
TGAGCCCNTG ACAAACCCCT NAGGATTTGT CGAATGTAAA GTCTCCNGAT
CTTCAATAAA NNTCCAAAAG NCTANCGAT
```

FIG. 27

PSGen 26-Novel

```
TCACTGGGCN NNNTGGTNGN CGTCATGCNN NAGGTTCCNN CCCCCNNANG
AACCTCCNGG TAATCTACAC NGGAGTCTTA AGTNGACAAN CCCACACTGC
GANGGTCAAG NGGATCACCA TCNCCNCCTC CCAAGCTTNT NCATTGATGC
TCTCTCTGTT CCGTNCCCTG CCGCTACACA TGGANGCTCT TNCTCCTTNT
CTCNTCTTAC NANNCAAACA TTGCCCTNTC TCATA
```

FIG. 28

PSGen 27-Novel

```
GGGAANGGGA NNAAAAAGGA ATTTTTTNGG GGGGGGNTTN TCTGGGAAAN
TTTTTTTTTT TTTTTGGNAA AAANGGGGGG GGAAANAANC CGNTTTTCCC
NAAAACNGGG GGGAACNGGC CGGGGGGGGA AAAAAAAGGG TTACNAAGGG
AAACCTTTNA AANNGGAANG GNTTTGCNNC CCTNTNGAAA NNTTTGCCCC
CCNNNAGGAA TCCCNGGNNA AACCCAANNC CNNCNCNCNG GGGGNCNNTN
CNANGGGACC CCAACNCGGG CCCNAACTNG GGGNAAANAN GGGCAAAACN
GGTNCCCGGG GNAAAANGGT ANCCCCTC
```

FIG. 29
PSGen 28-Novel

```
TGCCGAGCTG GGGGTGAAGC ACCGGAAAAC AACCGATCCA TCTCTTATCA
CAGGGTCTCC AAGATCCCAA ACCCAAAAGC CACATTGTTA ATTAGCCTTT
TTATTGTGTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT
TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTGGCAGC TCGGCA
```

FIG. 30
PSGen 29-Novel

```
TACGGGCGCT GATTTTTACG AACATTACCT GGCAGGGAAA TTTGATAAGT
ATCCACTGTG GGTGGCGCAC TACCTGGTAA AAGACAAACC CCGTGTGAAA
AGGCCCTGGA CTTTTTGGCA ACACAACGAA ACCGGCCACG TGAATGGCAT
CCGGTCTTAT GTGGACTTCA ATGTTTTCAA CGGGGACAGC ACAGATTTTG
CCGAACTATT AATGAAATAA TGCAGAATTT CGCTTTTCAA ATAAGCCCAT
GGATCCTGAC GTAAAATATT TCCTGCTGGT GATCGTGCAG TCCATTTCGA
TGCTCATACT TTGGCTGATG CTCAACATGA CCTTTGGGAT CTATTTTAAT
TTTGCTTTCC CCGACAATGG TTTGACGCTT GGCAACATCA TTTATTACCT
CTTCCTGCTG GGCAGCTCGG CA
```

FIG. 31
PEGen 32-Novel

```
TNCATANGCC CTGAGGTGGG GACGAAGCCC GAGTCCGTCC TGACATGTTT
CCAGTGGAAA AGATTTTGTT NTGAGCGTTN CTTTCTNNTT TNTTTTNNNT
TGNTTGTTNN ATGTTTTTGT TGTTGTTTTN TTNAAACTGT NTGTTGNCAN
TTCAACATNA ANGGNAGGNA ANTNTGTGNC TNCNTTGCAN TGTNNCATGN
TNCCCANANC CCAAAAAAAA AAAAAAAAAA AAAAAGAGTA CAAATATCAC
AAAATTTGAC ATTTTTGTAA TAATACTTTG GTTGTTGTTT GGTGACGGCG
ATTG
```

FIG. 32
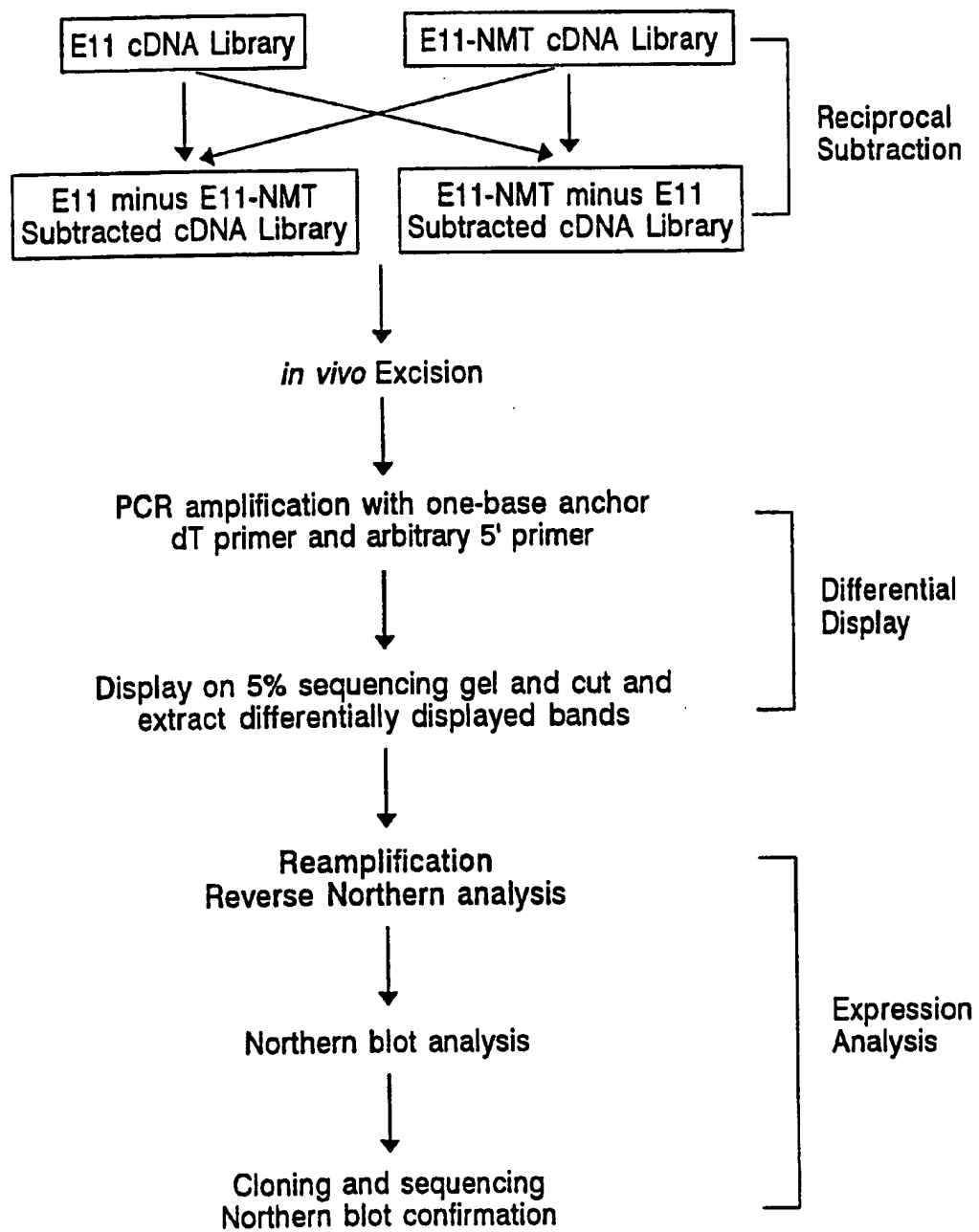
FIG. 33A   FIG. 33B
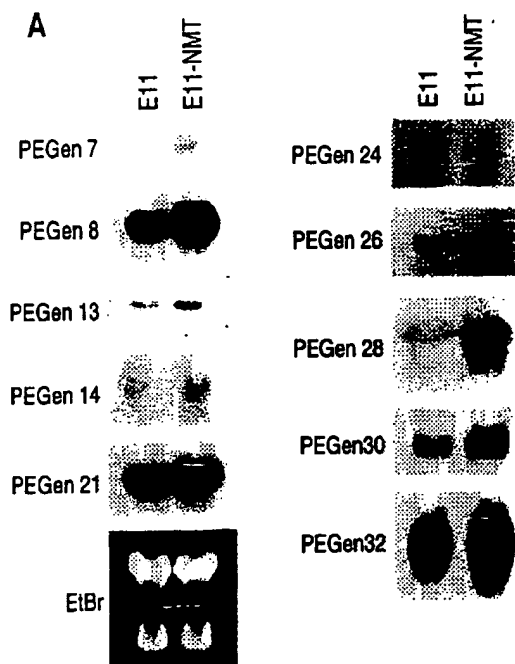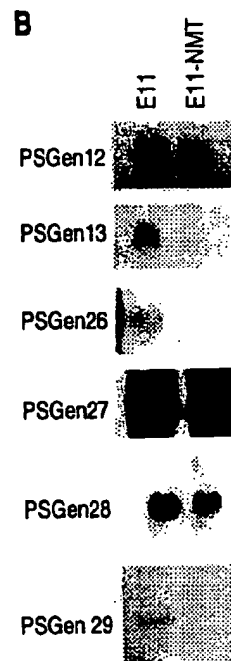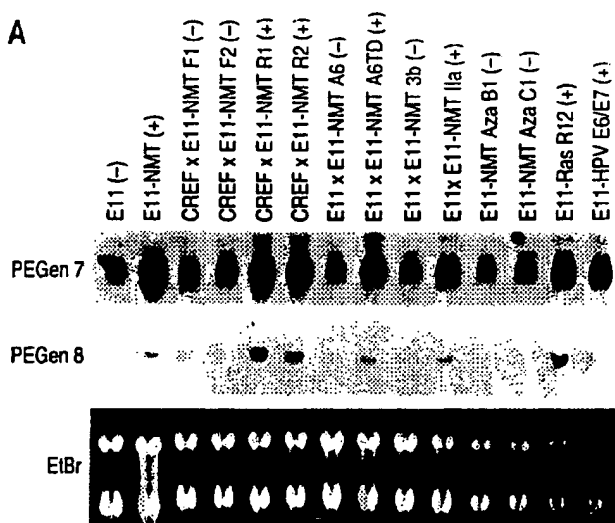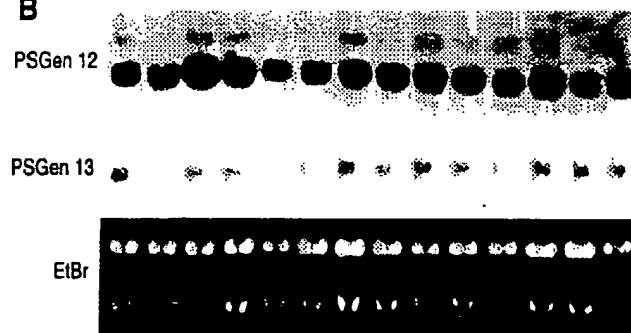
FIG. 34A A

FIG. 34B B

FIG. 35A

PSGen 12 cDNA Sequence

```
GCGGTGGTGA CGGTAGTATG GCCGCACTTT ATGGTGGCGT GGAAGGGGGA
GGCACACGGT CCAAAGTCCT TTTACTTTCT GAGGATGGGC AGATCCTGGC
AGAAGCAGAT GGACTGAGCA CAAATCACTG GCTGATTGGC ACAGGTACCT
GTGTGGAGAG GATCAATGAG ATGGTGGACA GGGCTAAACG GAAGGCTGGA
GTGGATCCTC TGGTACCCCT TCGAAGCCTG GGCTTGTCCC TGAGTGGTGG
GGAGCAGGAG GATGCAGTGA GGCTCCTGAT GGAGGAGTTG AGGGACCGAT
TTCCCTACCT GAGTGAAAGT TACTTCATCA CCACTGATGC AGCAGGTTCC
ATCGCCACAG CTACACCGGA TGGTGGGATT GTGCTCATCT CTGGAACAGG
CTCCAACTGT AGGCTTATCA ACCCTGATGG CTCTGAGAGT GGCTGTGGTG
GCTGGGGCCA CATGATGGGA GACGAGGGAT CAGCCTACTG GATTGCACAC
CAAGCTGTGA AAATTGTGTT TGACTCCATT GACAACCTGG AAGCAGCTCC
TCATGATATT GGCCATGTCA AGCAGGCCAT GTTCAACTAC TTCCAGGTGC
CAGATCGGCT AGGAATCCTC ACTCACTTGT ATAGGGACTT TGATAAGTCC
AAGTTTGCTG GATTTTGTCA GAAAATTGCA GAAGGTGCAC AGCAGGGAGA
CCCTCTTTCC AGGTTCATCT TCAGAAAGGC TGGGGAGATG CTGGGCAGAC
ACGTTGTGGC AGTATTGCCA GAGATTGACC CAGTTTTGTT CCAAGGGGAG
CTTGGCCTCC CCATTCTGTG TGTGGGCTCA GTGTGGAAGA GCTGGGAGCT
ACTGAAGGAA GGCTTTCTCC TGGCACTGAC GCAGGGCCGA GAGCAACAGG
CACAGAACTC CTTCTCCAGT TTCACCCTGA TGAAGTTGAG GCACTCTTCT
GCACTGGGAG GGGCCAGCCT GGGGGCCAGG CACATTGGAC ACCACCTTCC
CATGGACTAC AGCGTCAATG CCATTGCCTT CTATTCCTAT ACCTTCTAGG
GGCTGCCCCT CTTCCCATTC AGCCAACACT GAGTGTTGGG AGATTTCTCT
TTTTTAAAAA CACATGAGAA AATAAATGCA CTTTACTCCC TCCCCAAAAA
AAAAAAAAAA AAAAAAAAAA AAAA
```

PSGen 12 Protein Sequence

```
GGDGSMAALY GGVEGGGTRS KVLLLSEDGQ ILAEADGLST NHWLIGTGTC
VERINEMVDR AKRKAGVDPL VPLRSLGLSL SGGEQEDAVR LLMEELRDRF
PYLSESYFIT TDAAGSIATA TPDGGIVLIS GTGSNCRLIN PDGSESGCGG
WGHMMGDEGS AYWIAHQAVK IVFDSIDNLE AAPHDIGHVK QAMFNYFQVP
DRLGILTHLY RDFDKSKFAG FCQKIAEGAQ QGDPLSRFIF RKAGEMLGRH
VVAVLPEIDP VLFQGELGLP ILCVGSVWKS WELLKEGFLL ALTQGREQQA
QNSFSSFTLM KLRHSSALGG ASLGARHIGH HLPMDYSVNA IAFYSYTF·
```

FIG. 35B

PSGen 13 cDNA Sequence

```
GGCACGAGCT CTCCTCGTCC CCTCCCTTCT CCACTGCAGC CTTTCTCTTA
GCCCGAACCA CTTCCTTCTT CTGCTTGTTC CTCCCTAGGG CGCGGAAGCT
GAGTGCAGGG TTCAGACCCA CGCGGCGAGC AGCTCTTCAG TGAAGAAGGA
AGCAATCGGA GGGTCAGCAA TGAACGTGGA GCATGAGGTT AACCTCCTGG
TGGAGGAAAT TCATCGTCTG GGTTCCAAAA ATGCCGATGG GAAACTGAGT
GTGAAGTTTG GGGTCCTCTT CCAAGACGAC AGATGTGCCA ATCTCTTTGA
AACCGTTGGT GGGAACTCTG AAAGCCCGCA AAACGAAGGA AGATTGTTAC
GTACGCAGAA GAGCTGCTTT TGCAAGGTGT TCATGATGAT GTTGACATTG
TATTGCTGCA AGATTAATGT GGTTTGCAGA TCTGGGGGTA TCTGGTAAAC
TGGAATAATT AAGTTAAAGG ACAAACATGA AGTTCCTTAT GTATTTTTAT
AGACCTTTGT AAACAAAAGG GGACTTGTTG AGAAGTCCTG TTTTTATACC
TTGGAGCAAA ACATTACAAT GTAAAAATAA ACAAAACCTG TTATTTTTTT
TTTCTTAAGA AGGTAATCGG GAGACGTAGG CAATAAAATG TTTTCAGAGG
TGCGAAAAAG CTTTTGTTTT CTTAAACCAT TCTTAGTCTC TGCCACACTT
GACACTCCGT CAAAGTGAGA AGCGAACTAA AGACCAACTG CGGTGGAAAA
TATTATGTTT ATGTAATAAA AAAAAATCAT GTAAAAAAAA AAAAAAAAA
```

PSGen 13 Protein Sequence

```
MNVEHEVNLL VEEIHRLGSK NADGKLSVKF GVLFQDDRCA NLFETVGGNS
ESPQNEGRLL RTQKSCFCKV FMMMLTLYCC KINVVCRSGG IW·
```

FIG. 35C

PEGen 28 cDNA Sequence

```
GTGTGGTGTG TCTCTCAGAC GTCCGTGACA CTTTGATCCT GCCCTGCCGG
CACCTGTGCC TCTGCAACAC CTGTGCAGAC ACCCTGCGCT ACCAGGCCAA
CAACTGCCCC ATCTGCCGGC TGCCCTTCCG GGCACTGCTT CAGATCCGAG
CCATGAGGAA AAAAATTGGGC CCTCTGTCTC CAAGCAGCTT TAACCCCATC
ATCTCTTCCC AGACTTCGGA CTCTGAGGAA CATTCATCCT CAGAGAACAT
CCCTGCGGGC TATGAAGTGG TGTCTCTCCT GGAGGCCCTC AATGGGCCCC
TCACCTCATC CCCAGCGGTG CCTCCCCTTC ACGTTCTTGG AGATGGCCAC
CTCTCAGGAA TGCTGCCGTC CTATGGCAGT GATGGCCACC TGCCCCCTGT
TAGGACACTG TCCCCCCTTG ACCACCTGTC TGATTGCAAC AGCCAAGGGC
TCAAACTCAA CAAGTCTCTC TCCAAGTCCA TTTCCCAGAA TTCTTCTGTG
CTTCACGAAG AGGAAGATGA GCGCTCTTGC AGTGAGTCAG ACACTCAGCT
CTCTCAGAGG CTGTCAGCCC AGCATCCTGA AGAGGGACCT GATGTGACTC
CAGAGAGTGA GAACCTCACG CTGTCCTCCT CAGGGGCTGT TGACCAGTCA
TNTTGCACAG GGACTCCGCT CTCTTCCACC ATCTCCTCCC CAGAAGACCC
AGCCAGCAGC AGCCTGGCCC AGTCAGTCAT GTCCATGGCC TCCTCCCAGA
TCAGCACTGA CACCGTGTCC TCCATGTCTG GCTCCTACAT TGCACCTGGC
ACAGAAGAAG AAGGAGAGGC CCCACCTTCC CCCCGAGCTG CTAGCAGGGC
CCCTTCAGAA GAGGAGGAGA CCCCAGCAGA GTCCCCAGAC AGCAATTTTG
CTGGCCTTCC AGCTGGAGAG CAGGATGCAG AGGGAAATGA TATCATGGAG
GAAGAGGACA GATCCCCTGT GCAAGAAGAT GGCCAGAGGA CATGCGCATT
TCTAGGCATG GAGTGTGACA ATAACAATGA CTTTGACGTC GCGAGCGTGA
AAGCACTGGA CAATAAGCTG TGCTCTGAGG TCTGCTTACC CGGTACCTGG
CAACATGATG CCGCCATTAT CAACCGTCAC AATACCCAGC GCCGGCGACT
ATCACCCAGC AGCCTGGAGG ACCCTGAGGA GGACAGGCCT TGCGTATGGG
ATCCTTTGGC TGTCTGAGGG CACTGGCACC TGTACCTGGG CTTCCCCTCC
TGTCCGCCTT CCATCTGTCC TCACTGGACC ACAGGCCTTC TGGGCATCTT
CAACAAGACA CGTGGACTTT CTACTCTCAT GAAGGGAGGA CAGTGCAACC
CTCCACCAAC TTCATCTCCT GTAACCATGA TTCTTACCCT CTCAGAAAGT
ACCAGAAGCC TTCCTCCTGT GGGCTGATGT GTGCCAGCCA AACCCAGTGG
GTCAGCTGAG CTGAGGGTCA GGGCTGGTTG TTTCTGTAGC CTTTTCTCTT
CCAAATGGAG ACCAACGAGA AANAAAAAAA AAAAAAAA
```

PEGen 28 Protein Sequence

```
VVCLSDVRDT LILPCRHLCL CNTCADTLRY QANNCPICRL PFRALLQIRA
MRKKLGPLSP SSFNPIISSQ TSDSEEHSSS ENIPAGYEVV SLLEALNGPL
TSSPAVPPLH VLGDGHLSGM LPSYGSDGHL PPVRTLSPLD HLSDCNSQGL
KLNKSLSKSI SQNSSVLHEE EDERSCSESD TQLSQRLSAQ HPEEGPDVTP
ESENLTLSSS GAVDQSXCTG TPLSSTISSP EDPASSSLAQ SVMSMASSQI
STDTVSSMSG SYIAPGTEEE GEAPPSPRAA SRAPSEEEET PAESPDSNFA
GLPAGEQDAE GNDIMEEEDR SPVQEDGQRT CAFLGMECDN NNDFDVASVK
ALDNKLCSEV CLPGTWQHDA AIINRHNTQR RRLSPSSLED PEEDRPCVWD
PLAV·
```

FIG. 35D

PEGen 32 cDNA Sequence

```
GGCACGAGGC GCCGCCTTCC TGCTCGCGCC CTATCGCCGC CTTCCTGCTC
GCGCCCTATC GCCGCCTCCG AGTCTTCCTG CGCCCCGGGC TTCCGCCGCT
TCATTGATTT CCGTTTCTCG CCGCTGCAGC CTCCTGACAC GGTGATCCGG
GCGGGCCCCG CAGGAATTTT ATCCCCTCAC CGGCCTCACA CTAGTGTCGC
ATGTCCACTA TCCAGAACCT CCAATCTTTC GACCCCTTTG CTGATGCAAC
TAAGGGCGAC GACTTACTCC CGGCAGGGAC TGAGGACTAC ATTCATATAA
GAATCCAGCA GCGGAACGGC AGGAAGACGC TGACCACTGT GCAGGGCATT
GCGGACGATT ATGACAAAAA GAAACTTGTG AAAGCTTTCA AAAAGAAATT
CGCCTGTAAT GGGACTGTGA TTGAACACCC TGAGTACGGA GAGGTCATTC
AGCTTCAAGG CGACCAAAGG AAGAACATTT GCCAGTTTCT TTTGGAGGTT
GGCATCGTCA AGGAGGAGCA GCTGAAGGTT CACGGATTCT AAGATGAACC
CGAACATGTG GCGAGTTTCT TAAATGGTTT TGTTGTCTAA CTCAGTTTGG
CTGCCTCGGG AGATGATTCT TTACAGTAAA CGACAGACTT TGCGTTTATT
AAATCATTCA GACTTCCACT CACGCCTGCA TGGCTACAGA AAACATGGGG
TATGTAGGCT CCTAAGTCAC AAGGAAATCG CCGTGAGGTG GGGACGAAGC
CCGAGTCCGT CCTGACATGT TTCCAGTGGA AAAGATTTTG TTCTGAGCGT
TCATTTCTAG TTTATTTTCA CTTGATTGTT AAATGTTTTT GTTGTTGTTT
TATTAAACCA TGTATGTTGC AGCTTAACAA TAAAGGAGGA AAGTCTGTGC
GTCAAAAAAA AAAAAAAAA AA
```

PEGen 32 Protein Sequence

```
MSTIQNLQSF DPFADATKGD DLLPAGTEDY IHIRIQQRNG RKTLTTVQGI
ADDYDKKKLV KAFKKKFACN GTVIEHPEYG EVIQLQGDQR KNICQFLLEV
GIVKEEQLKV HGF·
```

FIG. 35E

PEGen 42 cDNA Sequence

```
GGCGTTGCGA CGTGGACATG TCGGCGTCGT TGGTCCGCGC CACCGTGCGG
GCCGTGAGCA AGAGAAAACT GCAACCCACG CGGGCGGCGC TCACGCTGAC
CCCCTCTGCT GTGAACAAGA TAAAACAACT TCTTAAAGAC AAGCCTGAGC
ATGTGGGTCT GAAAGTGGGT GTGCGGACCA GGGGCTGTAA CGGCCTCTCT
TACAGCCTGG AGTATACAAA GACAAAGGA GATGCTGATG AAGAAGTTAT
TCAAGACGGA GTCCGAGTGT TCATCGAGAA GAAAGCCCAG CTAACCCTGT
TAGGCACAGA GATGGACTAT GTGGAAGACA AACTGTCCAG TGAGTTTGTG
TTCAACAACC CCAACATCAA GGGAACCTGT GGCTGCGGTG AAAGCTTTAA
CGTCTGAAAG CTGAGGACTG CAAACTCCAG GAGAGCTGGG TCTGCCTTGG
AGCACACCGA AGAAATCATG TGATGTCCCG TGTCGGAAGT TAGTGTGTGG
CTGCCTCGTG GTTGAGAATA AAGTGAAGCA TTGAAAATCA AGCCAGCGTG
TTAGAGTTCC AAAAACATGG TGTCTGTTCT CTGTAAGACA CAAATGGAGA
GAACATGGTG TCTGTTCTCT GGAGGACACA AACTGAGAAA CTGTTGAGTC
CTCTGTCCTG TACAGAAAAC TCCTACCCTG CCCTTACGCT GTAGCCTGCT
CTGTGCTAGA ACCAGCTTCG TGACCATTGC TTTGCTGGGA ATTGAGGAAT
GGGATAACGG GTGTGCACCT GGGTCACAGA ATGGCTTGAG ACTGTCTCCT
GGCCCTGTCT CACCTCAGGC AGGGCAGCTG TGGGAGCAGC AGCTGTGGGA
GCGGTGAGGG GACCTGGTTT CCCTCACCTG TGGCGTGGCC CGTTGCATCT
TTACCACGTG CCTGTTGTCA GATACCTCAT TTGCCAGCCT CCAGCAAGCT
CAGCTATGAG TGCCAGTCTC AGGAGGTAGG GATCACGGGC CTGGTGTCAG
TCTGTCCTCT GGGGCGTGCT TCATGCGGTT TGCTTAGACC TTTCAGTTAG
AAGCGCTTGT GATGAGCAGC CAGGTAGACC TGCTGAGAGC GTGGTTCTCA
GAGCTTCTGC CCAGCCCTCC TCACAGGTCA CAGCAGACAG TGCTGTCTGA
GACACTCGGT GAGGAGACAT CCTGCCTGGC CAGTGCTCTT ACCAGTTTAG
AGACTGCATT AGTTTTCTCT TGAATGGAAG CCTTGTGTAA ACCCTTTTGT
CTGAATGGCC ATCCTGTTTA GAGCTTTGAA CCAGTAGTGT CTTCCTTCAG
AAGATCTGCA GCAGAGGGGT CCCTCTCAGC ACGGCACCTG GGGGGCAGAA
CATGCACACA CTTACAGTTG CCAGGGTGCA GATGCTCCCT GCTTCCCAGA
GGAAGCTTCT AAGTTTCTTT AATGTGGTCA TCACCAGTTT TTTGAGCCAT
GGTTTTGCTG TATACTACAG GCCAGCCTTG AACCCACAAC AATCCTCCTG
CTTCCACGTT CAGAGGCATG TGCTACCACA CCTGACCTGG ATCCCAAGTT
TCTCTTTAAG TGGTCTTGAT GGACTTGGGT CGGACATCTT AGTGACCTGT
GAATTCTTCT GTGGAGGCTG AGTCTCACGT AGCCGAGTTT AATATCTGTG
CTATTTACTA AAGTATCTGC CACCAAATTG TACCAACTCA TAGTTTTATA
TGAATGTTGA TGAGTCTGTA TCATAAATAG AATTGTTGAT ACATCCTTAA
TTTGTGCAAT ATTGTATGAA GAAGATTGTT ATCAATTAAA ACCACGCCTC
TTTATGATCC TAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA
AAAAAAA
```

PEGen 42 Protein Sequence

```
RCDVDMSASL VRATVRAVSK RKLQPTRAAL TLTPSAVNKI KQLLKDKPEH
VGLKVGVRTR GCNGLSYSLE YTKTKGDADE EVIQDGVRVF IEKKAQLTLL
GTEMDYVEDK LSSEFVFNNP NIKGTCGCGE SFNV·
```

FIG. 35F

PEGen 45 cDNA Sequence

```
ACGAGCTGAA GGTCACTTCG CGCACGGGTT GGACCTGGGG CAGGTTGGAG
GAGTAGGAGT ATGTCATTGG GCGCGAAGAC GGGGTCTGGG GCAAAAAAGA
AGGGAGGCTG GAGAAATCTG GACCCGAGAC GTAGTAAGTA CAACTTGGCA
AATACATGTT AGAGGAGCAG GGACCACGCT CATCAAAATC CATCATTGGG
CTACCTTGGG CTCTCCGCAG TAGCCGAGCT TAACATGATT CTCCACTGCA
GCTGCCTCTT TGAAGCGGAT CCGTGAAGTA GAAATTTGGA GACGTAAGCT
GACGTGGAAA TCTATCCCCA TCCTTAGCAG GGAGGTGCTG GTCATGTGAC
CCGATGTTGA AATTGACAAG CCGCGAGCTA GTCCCGGCTT TTTTTTTTTA
ACCCCCCTCC CTTTCCTTTT TTCCCCCTCC CCTCCCTCCT CGGCTTCCTT
TCTTTGTAGC CACCTCAGGG GAAGCAACAG ATCGTCACTC GGTGTTCTCA
CCGAAAGCAC GTAATCGCCG GTGTAACTCA TGTTGGCTGG GGGGCCTCCC
CGCTCGCAGA AAGGCTGGGG TGCGCCCCCA AGCAGCTTTC CTTTGCTCAG
CTGCATGGTC CTGGTCCACG AGCGCTCTGA GGGCGGCAAG AGAGCGCAAC
TCCTGACGCC TCCCCCCACT CCCCGGTGGG TGAGGGATGC TCTGGGATGG
GGGTGGCCAG GTGAACGCCC GGAATTGTGT AGCTTCAGGT TCCGGAGTCT
GTTGTCCGAA GGCTTACGTT CAGCACCTTC TTCGCAGTCC CCCTCCCACA
GACTTGCTCT GGAAAGCACC TCAGTCTCAG AATCTGGCTG GACCCCATTT
GGGGCCAGGC TTCGCAGCCA CGATGTGCCG GGCTTCGTGG CTTGTCCGAT
TTGCACGGTG ACTTGATTAC ACGCTCTCAT TCATGGTCAC TTCCGAAGCG
CTTTAGTGCC TTCCGTCCCC AAACCGCCAA CAGGCAAAGC GGCTTTCCTC
CGCGGTTTGT CAATAATCCG CGCTGTCCGG AAGGGCTTCG CCTTACCCGG
GTTCCACCTT CCCTGTATCT TTCTGCTTAC TTCCTCATCC CACACTCTGT
CCTTGGAGGA ACCCCTTCTC CTCGCTGCCT GTAGGGGTTC GGAGTGACTC
CACAGAGCCA GAGGCGCTTC TGCTCACCGG TCCGCAAGCT GCCTGGTCTG
CTGAAGCTGA CGAATCGGGA AACCATGCAA TTGAGGCGAA CCTTGGGCTG
CTTTAGAGGC GCTGAGGAGC CTTCTCCTGG GAGGCCCAAG GTCGATTTCA
GCCCACCAGG ATCTGGGGAA GACCCAACTA GGGGTAAGAG CACACCGGAA
GGCCAAGTCC GAGTTCCAGT CCTAGAAGAG GCGGCTGCGG GCAAGGTTAT
GACATTGGCC CTGGACACTG GTTTCCCAGG AGCTATTCTT TCTCAAGAAC
TCCACAGCAC GGGGCTGTCT CCAGAAAATA CTCTTCAACG TTTATTTCCT
TTAATCGTCA ACCCGCAGCC CTACGGCGGT TAATGCGAGA GGCCAAAAAT
GTTTGGAGGA AGAAAAACAA AGGCAGGAAG TGGCCGCGGC CTGACGGTGC
GTGTGTGTCT GTAAAGAAGG GAGGGAGCCG GTTCAATCTC TTCTTTTTTT
CCCCGAATTT CAAGGTTTAG GCAGACCCCC GTAGGGCCTG GCCGAGGCTC
ACCCGGCGGA GCATTTGGAG GTGGCCAATG AGTAAGGCTC GTCGGGCTGA
AAGGCTAAGA AGGAGATTTG ATCGGCAGAA CAAACCAAGC CTTTTTGGAG
GTTTCTTCTG ATTTGGTCCT AAAGGGTATA TGCTAGTGTC CACAGCGGCT
CCTGTGGCTG CTGTTTTCCT CCTGTCGGAC TAAATGTACC AAGAAGGGAG
AGAGATTGAG GCACCTTGCG CGCTCCTCTC TCCTTCCGAG GTAGAATATC
AGAATAAAGT GTATTCAGGT GCCAA
```

FIG. 35G-1

PEGen 50 cDNA Sequence

A:

```
ATCGGGCTGT ACTAACAGAT TGTTTGTAAA CAGTGACACA GTGATAACTT
CCGTGTTACT TCTTAACTTT ATGTTTCTGC TTTCAGATCT CCCTCCCCTT
CCAGAGGAAG TTAGCGATGC CATAGCTTTA ATGTCTGTTT TAGCTGCAAA
ACTCATTGTT CACTTTCTGT TAGAAAATCT AAAGCAGGTG GTATGCAATT
TCTCTTGATT TGGAATTCTT TAAAGGCAAG TAAATTTGGA ACTCCTGTGT
TGGGGGGTTA ACGGAGGTAG GAACCCAATG GTGTGTCCCT AGGTCGTCCC
CGTTCTCGGA TAGCACAGTC TGCATAGCCA TAGCTCTCAA TTATGTCACT
ACCCTAATCA TCGCAGCCCG GTTCTCACGG ACTCTTTGAA GTCCCAAAAT
GACTTTTGTT TGATCCTGAT TTGGATTTTC AATGGAAAGT AAAAGCTTGG
GGTGAGGAAG CAGCAGCTAA AGCAGGGAGT TGAGCCAGTG AATTGCTGAC
GGAAAGGATT CTGGTCTTGG AGGAGGGGGA CCTGAAGCAG AAGGAAAAGG
GATCCTTCGC TTAAGTTCTT AGGAAAAATC TTGACTCAGA ATCCCAAGAT
TTTTCCCTTC ATCCCAGCCG GGTAAATATT TGGTTTTGTC TTTTAAGTAT
AGCATGAAGC CCGTGGATGA GAGCCATGTG TTGTAGGATT CTCTTCCCTA
TTGGCTCTGA GCTTGTGTCA CCGTATCAGT TTGCTCCCTA CAAAGGGACC
TAGTTTGGAA AGGATTGGAA GGGCAACTGT TCAGCGGCAA TGGAACACCC
AAACGTGGAC TGGGACAACG GGATTCTGAT AAAGGGAAAT TTCTGGTCTG
GTCCTGGCTG TGTCATAGCT CTTTATGTGT GCATGGAGAG CTCTTGATCC
AAGTAGAATA TGTAACAATA CAGACCAGGA TCTTCCAGTC AGTACTGCTG
GGTGGAAGTG GGCGGGTGAT GGTAGTTGCT AGAAGAATCA TTAAGACAGC
ATCTGCGGTG AATGCGTCCC AAAGCCTCGC GGCATCAGTT TCATCTCTAA
ACCATTAGCT TACAGTTGAT TCCGTTTCCT GGGACAGAGA AACATCCCCA
CGCGAAGTGA CTGTGTTGTG TATTCATAGC ACTGCAAATA AATTCACGCG
CCATGATGAA ACCTTGCAAA TACGCTTTGA CCAAAAAAAA AAAAAA
```

FIG. 35G-2

B:

```
GGGTGTGGGG CAGCTGGGTG GGAGCAGCGT GCAGGCTACC AGCACCAAGT
GGTGTGCCTC TCCGGGGGTG TGTGCAGAAG GCTCCTGGGG AAAACTGCAC
AGGTACCACC CCTAGACAGA AATCGAAAAC CCACTTCTCT CGGTGCCCCA
AGCAATACAA GCATTACTGC ATCCATGGGA GATGCCGCTT CGTGATGGAC
GAACAAACTC CCTCCTGCAT CTGTGAGATA GGCTACTTTG GGGCCCGGTG
TGAGCAGGTG GACCTGTTTT ATCTCCAGCA GGACAGGGGG CAGATCCTGG
TGGTCTGCTT GATAGGCGTC ATGGTGCTGT TCATCATTTT AGTCATTGGC
GTCTTGCACC TGCTGTCATC CTCTTCGGAA ACATCGCAAA AAGAAGAAGG
AAGAGAAAAT GGAAACTTTG AGTAAAGATA AAACTCCCAT AAGTGAAGAT
ATTCAAGAGA CCAATATTGC TTAACTTAAT GATTATAAAG TTACCACAAG
CTGATGGCGA GCTCCAAAAG ACCTGACTCA TTTGCAGATG GACAGGACAT
GTCTCAGGAA AACAGCTTGC AGAAATGAAT GTTTAAATAT TGTATTTGCT
TTTTCATTTT ATTTGTAACT GTGTGTTGTT ATTGTTTTTA ATAATGATAT
TTTTGTTACA GTCTGATAGC TGAGAAAAAA ATGACCTGGT TAGGTGACGA
CAATAAGGGA CATTGAATAT AAACTTTGTT GCTAGGATTA TTAAACAAAC
AAAATTTGGA AAGAAGTTAG ATTTTAAGAA CTGAGTCATG GTCAGGCAGC
GATGGCACAC ATCTTTAATC CCAGCACTTG GGAGCAGAGG CAGGTAGATC
TCTGGGAGTT TGAGGTCAGC CTGGTCTACA AAGCAAGATC CAGGGTAGCC
AAGGTTATAT AGAGAAACCC TGTCTCACAA AACCAAACCA ACCAATCAAC
CAAACAGCAA AACACCTGAG TCGATAAAAG GGCTCCCCAG GTTTATACAC
TTACCGTATG CTAAGAGCTT GAAATATATT GTTTCGTTTT ATCGTTCAGT
AGTCTGTGAG ATTGCATTTT TTCTCATTCC TATATATAAA AAAGTTAAAT
GATTTCCCTT AGATGTAGAG ATAGAGGAAG TTAGCGATGC CATAGCTTT
```

FIG. 36

PSGen 27-Novel

```
NTCNNCTTNN CNNNGGCTGA TATCNGGCNC TTCNTCCNCG ATCNCAGATA
CNNGCNCACC GGNNNTNTCN GNGGTNATCN TCCNCCATCT CTCNTCCCCG
ACNTGCACTC CGGGTNTNNT ACACNGGACA CTGTATCNNA CAGNAAACCT
NCCCNGGCCC CAGGGATCAC CATNCCTCGN CCCNGCNTGT NTATAANATC
AGGNNNTACA TCNANGAACN NACTATCACN GNTCTCTNTT NNCTCAGTGT
NCACCTTCCA CTNCNGAANC TNNTCGCTNC NCCNCNGTTG GGAAAGGCGA
NCNGTNCCGG CNACATGCCG TTTNCGNCNT CTGNNCACNT GGGGATCTNC
TNCAANGNAA TCAATTNGNG TAACCCACGG TTTNCNCAAT CACTACTTCT
CANNCNANGG CCNTTGAANT GTTATCCCAC CACCANGGGG CNANTCGGGA
CCTNACAATT CATCCTCAGC CGGCCCCAGN CTTAAAAAAT TCAAAGGNCN
CTTGCCCGCN TTNTTNCCTT AGCCCGCCNC CNGACAACAN CCNANNAACA
ACCCCCNNTC TTANGTTGCN NANCCCACAG GANNTTGNNA TACCGGGTTT
CCCCNGAAAC TNCTCAANGC CNCCGTTCCA ACCCCCGTTA CGAAACCGTN
CCCNTTTCCT TCCGAGNTTG CCTATTAANN CCCCCNAAGT TCTNCTTCGT
TNGNTTCCTC CGAAANG
```

-1-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: The Trustees of Columbia University in the City of New York

    (ii) TITLE OF INVENTION: RECIPROCAL SUBTRACTION DIFFERENTIAL
         DISPLAY

    (iii) NUMBER OF SEQUENCES: 24

    (iv) CORRESPONDENCE ADDRESS:
         (A) ADDRESSEE: Cooper & Dunham LLP
         (B) STREET: 1185 Avenue of the Americas
         (C) CITY: New York
         (D) STATE: New York
         (E) COUNTRY: USA
         (F) ZIP: 10036

    (v) COMPUTER READABLE FORM:
         (A) MEDIUM TYPE: Floppy disk
         (B) COMPUTER: IBM PC compatible
         (C) OPERATING SYSTEM: PC-DOS/MS-DOS
         (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

    (vi) CURRENT APPLICATION DATA:
         (A) APPLICATION NUMBER:
         (B) FILING DATE:
         (C) CLASSIFICATION:

    (viii) ATTORNEY/AGENT INFORMATION:
         (A) NAME: White, John P.
         (B) REGISTRATION NUMBER: 28,678
         (C) REFERENCE/DOCKET NUMBER: 55551-C-PCT/JPW/AKC

    (ix) TELECOMMUNICATION INFORMATION:
         (A) TELEPHONE: (212) 278-0400
         (B) TELEFAX: (212) 391-0525

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 371 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: not relevant
         (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

5   TAAANCGGTG GTACTGCTGC ACGGTCCTCC GGGTACTGGA AAGACATCCC TTTGTAAGGC   60

ATTAGCCCAG AAACTGACCA TCAGACTGTC AANCAGGTAC CGGTATGGCC AGTTAATTGA   120

AATAAACAGC CACAGCCTAT TTTCTAAGTG GTNTTCAGAA AGTGGCAAGT TGGTAACTAA   180

10   GATGTTCCAG AAGATTCANG ACTTGATTGA TGATAANNAA NCTTTGGTGT TTGTCCTGAT   240

TGATGANGTA AGCACTCANN GGTACTCATT CTTNGTCTGC ATTGCCTCTT GCTATTACTG   300

CCTGATCCCT CTCATTTGGT TCACTGTGTC GCNANCTCTT TTCTATGGAT CTTTTCCNAN   360

15   CCACCCGTTT C   371

20   (2) INFORMATION FOR SEQ ID NO:2:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 245 base pairs
            (B) TYPE: nucleic acid
25   (C) STRANDEDNESS: not relevant
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

30   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GTGACGTAGG GTCTGTTGCG TCAATGGTTA TAGCAAGTGA TGCTCTCTGA TTATTACTGC   60

TGACAATACT CGGCCAACAA TTCTTGCATA GAGTGCTGAT AAATAACTAT GTTACAAAAA   120

35   GGGGTGGTCC CTGGAGAACA TTACAGGCTT CCCTAGGTAA GTGTGCAGGT CAGGAGACGG   180

CATATTCAAT CAGATGGCTG ATAGTTCTCC GTGGTTATGC ACCGGCTCCA GCTTGCCTAC   240

40   GTCAC   245

     (2) INFORMATION FOR SEQ ID NO:3:

45   (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 178 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: not relevant

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        (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

5   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
GCAGCATGAT GAATTTAATG CAACAGTCAT AGCAGGGCAA GGGGAGAGAA AGGCAGATGG        60

ACTATCTGCA TCATCAAGCG AGGGCTTGTG TCGGCGGCTA TGTGCAGAGA CGAGCAGGGC       120

GAGGCACTTA AAAGCTGCTN GATGAAAATC CACCCAGGAG AANTCTGGGC CTACGTCA         178
```

10

   (2) INFORMATION FOR SEQ ID NO:4:

15

     (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 191 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: not relevant

20   (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

25

```
TGACGTAGGC CCAGACTTCT CCTGGGTGGA TTTTCATCCA GCAGCTTTTA AGTGCCTCGC        60

CCTGCTCGTC TCTGCACATA GCCGCCGACA CAAGCCCTCG CTTGATGATG CAGATAGTCC       120

ATCTGCCTTT CTCTCCCCTT GCCCTGCTAT GACTGTTGCA TTAAATTCAT CATGCTGCCA       180

AAAAAAAAAA A                                                           191
```

30

   (2) INFORMATION FOR SEQ ID NO:5:

35

     (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 124 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: not relevant

40   (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

45

```
GCCATAAATA CACTTTATTT CATTCGAAAT GCATAATCAC ACTGGGAGCA CTCCCTTTGG        60

AGCACTCCTC TAGCAGCAGG TCCGAAGTGC TCCAGCATCG TCAGCTGGCT CCAACACCTA       120
```

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```
CGTC                                                          124
```

(2) INFORMATION FOR SEQ ID NO:6:

5　　　　　　(i) SEQUENCE CHARACTERISTICS:
　　　　　　　　(A) LENGTH: 61 base pairs
　　　　　　　　(B) TYPE: nucleic acid
　　　　　　　　(C) STRANDEDNESS: not relevant
　　　　　　　　(D) TOPOLOGY: linear

10　　　　　(ii) MOLECULE TYPE: cDNA

　　　　　　(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
15   TTTTTTTTTT TTTGGAAACA GAATAAAGTG CTTTATTCTC TGGCTGGCTC TCCTACGTCA    60

     C                                                           61
```

(2) INFORMATION FOR SEQ ID NO:7:

20　　　　　(i) SEQUENCE CHARACTERISTICS:
　　　　　　　　(A) LENGTH: 216 base pairs
　　　　　　　　(B) TYPE: nucleic acid
　　　　　　　　(C) STRANDEDNESS: not relevant
25　　　　　　　(D) TOPOLOGY: linear

　　　　　　(ii) MOLECULE TYPE: cDNA

　　　　　　(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

30
```
     TCGGCGATAG CATTGGAGCA AGTCTTATCA GCAAGCAATG TTTTCAGTTA TGTTTCAAAG    60

     TTAAGAATGG GTTTAAACTT GCTGAACGTA AAGATTGACC CTCAAGTCAC TGTAGCTTTA   120

35   GTACTTGCTT ATTGTATTAG TTTANATGCT AGCACCGCAT GTGCTCTGCA TATTCTGGTT   180

     TTATTAAAAT AAAAAGTTGA ACTGCAAAAA AAAAAA                            216
```

(2) INFORMATION FOR SEQ ID NO:8:

40　　　　　(i) SEQUENCE CHARACTERISTICS:
　　　　　　　　(A) LENGTH: 334 base pairs
　　　　　　　　(B) TYPE: nucleic acid
　　　　　　　　(C) STRANDEDNESS: not relevant
45　　　　　　　(D) TOPOLOGY: linear

　　　　　　(ii) MOLECULE TYPE: cDNA

-5-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TNGCCAGGCT ATGTCTCAGA     60

CTTTATTATT ATTATTATTA TTATTATTAT TATAAATAAA ACATGTNCTT TCAATTAGGT    120

TACAANAGTA TTTATCTCCA TAACGCTTCT TCATACATCC TTAGTTTTGG ATTAAAGTAC    180

CATCCACCCC AACTCAAACT GTAACCCCCA GTAATCCCCT CTAACGTGGA AATTTCTGGT    240

TTAACAACTC AGTTAACTGC CCCACAAACA GTGGGAGGCC GCTCTTGCAT GGCTATGCCA    300

CGTAACCCTT CACTGCTTCA CTTCTTCGCT GGCT                                334
```

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 136 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
GACCGCTTGT ACCATCCAAC TTGCTTTGTC TTCTGCAGAG AGGAGGCTAA AGCCCTTGAG     60

CTGGCTGGCA CTGTACTCAG GCCGGAAGCC CAGCTCGTCC CGGTTCTTGA CAAAGCAAGT    120

TGGATGGTAC AAGCGG                                                    136
```

(2) INFORMATION FOR SEQ ID NO:10:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 316 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
TGCCGAGCTG GGTATTGTGA CGGTTGATAA TGGCGGCATC ATGTTGCCAG GTACCGGGTA     60

AGCAGACCTC AGAGCACAGC TTATTGTCCA GTGCTTTCAC GCTCGCGACG TCAAAGTCAT    120
```

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```
TGTTATTGTC ACACTCCATG CCTAGAAATG CGCATGTCCT CTGGCCATCT TCTTGCACAG      180

GGGATCTGTC CTCTTCCTCC ATGATATCAT TTCCCTCTGC ATCCTGCTCT CCAGCTGGAA      240

GGCCAGCAAA ATTGCTGTCT GGGGACTCTG CTGGGGTCTC CTCCTCTTCT GAAGGGGCCC      300

TGCTAGCAGC TCGGCA                                                      316
```

(2) INFORMATION FOR SEQ ID NO:11:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 337 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: not relevant
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
AGGGGTCTTG ATGGACTTGG GTCGGACATC TTAGTGACCT GTGAATTCTT CTGTGGAGGC       60

TGAGTCTCAC GTAGCCGAGT TTAATATCTG TGCTATTTAC TAAAGTATCT GCCACCAAAT      120

TGTACCAACT CATAGTTTTA TATGAATGTT GATGAGTCTG TATCATAAAT AGAATTGTTG      180

ATACATCCTT AATTTGTGCA ATATTGTATG AAGAAGATTG TTATCAATTA AAACCACGCC      240

TCTTTATGAT CCTNNNAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA      300

AACCNCCTCA AATCCATNGG TTCTAACCCA AAACCCT                               337
```

(2) INFORMATION FOR SEQ ID NO:12:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 307 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: not relevant
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
TTTTTTTTTT CATACACCAT CAAACCAATT TTATTTCTAT AGCAACGTTT CTCACGTCTG       60

AACCTGAGAA TAAGTCACCA GCTCTTGACA GTAAACATGG GCCCTATCAA ATTATATTAG      120
```

```
ACTCCTCAGT GTCCCGCCAT GTGGCCTTGC ACCAAATCAA TTAGTTTGAG GGCCAAAATC     180

CTGTTGGGTT TCAAATAAAG TGTCAGGTCA TAAGGAGGGG GAGGGACTCA ATTCATGGGA     240

ACATTTTTAC CTGTTCAAAT AGATAAACTG AATTGCCCTA TCTGTGGTCA CCTGGATCCA     300

AGACCCT                                                              307


(2) INFORMATION FOR SEQ ID NO:13:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 296 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CCCTGACGAT AAATGGTAAG GAACTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTNC      60

GAAATAAACA AACACAGCTT ATTATTTGGG GGAACATTAA NTTCTATAAN TGAACACAAA     120

ANAAAATTAA NANTTAATGG GGGGGTANAA GGGACTTTGA ATCTATCTGG TATCATGACA     180

TTGAAGCANA NACCTGANTG ACCAGAAAGA GAGAGAGAGA GAGAGAGAGA GAGAGAGAGA     240

GAGAGGTTTC ATATGAGCTA GTGTTACAGG CTTTATTAGT CTATTAGTCA GGGACC        296


(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 319 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AATCGGGCTG GATGGGTGTA TCCGGCACTG TTTCGTAGCG GCAGCAACTG GGTGCTTCTA      60

TCTGAAAGCG GGCTTCACAA AAACTACTGC GCCACCCGAC TCGCTGCGGC ATCGCCCGGT     120

GGCGAGTACC GTATCGCCTT TCCTGGTGCA GAAGAAGTGT TTACAGGAGG CGGTCATTTA     180
```

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```
      CCGCAATCTG ATTCTGTTTT TTATTCTCCC TGGCGGGTGA TCGCGATCGG CAGTTTGAAA      240

      ACGATCGTTG AATCCACGCT CGGGAATGAT GTGGCTTCGC CGCCAACGCT TACTGACATT      300

5     TCATTTGTAC AGCCCGATT                                                    319



      (2) INFORMATION FOR SEQ ID NO:15:

10              (i) SEQUENCE CHARACTERISTICS:
                    (A) LENGTH: 287 base pairs
                    (B) TYPE: nucleic acid
                    (C) STRANDEDNESS: not relevant
                    (D) TOPOLOGY: linear
15
                (ii) MOLECULE TYPE: cDNA


                (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:


20    GCCGAGCTGT GTAAAACCAT CTATCCTCTG GCAGATCTAC TTGCCAGGCC ACTCCCAGGG       60

      GGGGTAGACC CTCTAAAGCT TGAGATTTAT CTTACAGATG AAGACTTCGA GTTTGCACTC      120

      GACATGACCA GAGATGAATT CAACGCACTG CCCACCTGGA AGCAAATGAA CCTGAAGAAA      180
25
      GCGAAAGGCC TGTTCTGAGG GTGAGATGAC AGCCACAGAG AGGTCACTGC CACTAGACCA      240

      GAAAGTGGAT GGAGATATAT ATTTGGACTG GTGTTTTTTT CTGTCAG                    287


30
      (2) INFORMATION FOR SEQ ID NO:16:

                (i) SEQUENCE CHARACTERISTICS:
                    (A) LENGTH: 344 base pairs
35                  (B) TYPE: nucleic acid
                    (C) STRANDEDNESS: not relevant
                    (D) TOPOLOGY: linear


                (ii) MOLECULE TYPE: cDNA
40
                (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:


      ATCGGGCTGC AGATTGGAGA CAAGATCATG CAGGTGAACG GCTGGGACAT GACCATGGTC       60

45    ACTCATGACC AGGCTCGGAA GCGGCTCACC AAACGTTCGG AGGAAGTGGT CCGCCTGCTG      120

      GTGACTCGGC AGTCTCTGCA GAAGGCCGTA CAGCAGTCCA TGCTGTCATA GCTGTAGTCA      180
```

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```
GCCTAGACTT CTGCCCACTG ACCTTTTNGG GCACTGAGAA CACATCCACG CTCTGTCTGT      240

ATCTAGTTCT GGCTTCTGCT GTGTGCTANG CCCCAGCTCT GAGGAGTAAC AGCTGATCCC      300
```

5   `AAAGGTCCAA GCCAACCTTC TTACCCCTCA GCCCCCANCC CGAT                      344`

      (2) INFORMATION FOR SEQ ID NO:17:

10   (i) SEQUENCE CHARACTERISTICS:
                  (A) LENGTH: 300 base pairs
                  (B) TYPE: nucleic acid
                  (C) STRANDEDNESS: not relevant
                  (D) TOPOLOGY: linear

15
              (ii) MOLECULE TYPE: cDNA

              (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

20   `TTTTTTTTTT TTTGGGCAAC TATGTATTTA TTGTGTTTGG AAGGCAGAGT GAGGGAGGAG       60`

      `ACCCCAGCAG GAAGAAGACT GGGTGCAGTC TAGAGTTCCT AGTCAAGAGT AGGAAGGTTT      120`

      `CTGTTATACC CATCATAGAA CGAGAGAGGG GGCTCAATAG ATCATCCCCT TTGTCTCTCC      180`

25   `ACGGGGCTTC TTGAGCTTCT CAAAGTTCTT CAGGATGATG TCATATAACA CAGCATAAGC      240`

      `GTTACGGATC TCCATGACCA TCAGCCGGAT CTCCTGGTAT TCCGCCTCGT CCAGCTCGGC      300`

30
      (2) INFORMATION FOR SEQ ID NO:18:

              (i) SEQUENCE CHARACTERISTICS:
                  (A) LENGTH: 461 base pairs
35   (B) TYPE: nucleic acid
                  (C) STRANDEDNESS: not relevant
                  (D) TOPOLOGY: linear

              (ii) MOLECULE TYPE: cDNA
40

              (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

      `AANATCTGCT TAAAAGTTCT TTAATTTGTA CCATTTCTTC AAATAAAGAA TTTTGGTACA       60`
45
      `AATTAAAGAA CTTTTAAGCA GATGTTTTGG TGCAACTAAT AGAAAAGATA AAGGCAGCCT      120`

      `GACATGCATG CACTGCCTCA GTGACCAGTA AAGTCACATG NCCTTGGGAC GTCAGCTTAG      180`

-10-

NTTTATCACN GTGTCCCAGG GGTGCTTGTC AAAGAGATAT TCTGCCATGC CAGATTCAGG   240

GGCTCCCATC TTGCGTAAGT TGGTCACGTG GTCACCCAGT TCTTTAATGG ATTTCACCTG   300

5   CTCATTCAGG TAATGCGTCT CAATGAAGTC ACATAAGTGG GGATCATTCT TGTCAGTAGC   360

CAGTTTGTGA AGTTCCAGTA GTGACTGATT CACACTCTTT TCCAAGTGCA GTGCACACTC   420

CATTGCATTC AGCCCGCTCT CCCAGTCATC ACGGTCACNT A   461

10

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
15   (A) LENGTH: 280 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: not relevant
            (D) TOPOLOGY: linear

20   (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

25   TGACGTAGGG CCGAGAGCAA CAAGCACAGA ACTCCTTCTC CAGTTTCACC CTGATGAAGT   60

TGAGGCACTC TTCTGCACTG GGAGGGGCCA GCCTGGGGGC CAGGCACATT GGACACCACC   120

TTCCCATGGA CTACAGCGTC AATGCCATTG CCTTCTATTC CTATACCTTC TAGGGGCTGC   180

30   CCCTCTTCCC ATTCAGCCAA CACTGAGTGT TGGGAGATTT CTCTTTTTTA AAAACACATG   240

AGAAAATAAA TGCACTTTAC TCCCTCCCCA AAAAAAAAAA   280

35
(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 177 base pairs
40   (B) TYPE: nucleic acid
            (C) STRANDEDNESS: not relevant
            (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

-11-

```
         GTAGGCAATA AAATGTTTTC AGAGGTGCGA AAAAGCTTTT GTTTTCTTAA ACCATTCTTA        60

         GTCTCTGCCA CACTTGACAC TCCGTCAAAG TGAGAAGCGA ACTAAAGACC AACTGCGGTG       120

5        GAAAATATTA TGTTTATGTA ATAAAAAAAA ATCATGTAAC TGCAAAAAAA AAAAAAA          177
```


(2) INFORMATION FOR SEQ ID NO:21:

10   (i) SEQUENCE CHARACTERISTICS:
                  (A) LENGTH: 633 base pairs
                  (B) TYPE: nucleic acid
                  (C) STRANDEDNESS: not relevant
                  (D) TOPOLOGY: linear
15
            (ii) MOLECULE TYPE: cDNA

            (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:
20
```
         TGCCGAGCTG AAAACATACA TCCGCACCGG GTTGAGATAG CTGGCCCTCC GTCCCCGGGC        60

         ATACTCTTTG GATAAGAACC CCGGCCTTGT TACCAGGTAC CGGAGTGAGC TGAAAAATTT       120

25       ACCGTCGAAA TGGGTGATGT CCTGGAAAAA ATGGTTCACC AGCTGCCAGG CAGATTCTTT       180

         GGGTTCCACA TTTTCCTGCC CACAGATGTG GCAGAAGCGG TCAAGTAATG CAGCATTACA       240

         ATTGAGGCAG ATCTTTTCTT TTCTTTCCTT GGAGTGGCTC AACCAGCGAT TTTGGTTAAA       300

30       AATAATCAAA AAAGCGACGG CAAAACTTTT GTTATATTCC CGCCTGTGGC ATTTGAACTG       360

         TGCCCGGCAA CCGAATAACT TTTAATTTTG AAAATAAAAT GCATACTAGA TTTTTAGCGG       420

35       TTGCCTCCTG GCCATTGCTT CAGGCGCCNG CACAGCGTCA GCCCAGTTTT ACCACNANGA       480

         ATATCCTAAG CGTTGAAACA GGGCACAGCC GAAAAAAACN CTGGCNACAA AAAANATCCG       540

         GACATCCTTT TTCCAATTTT GAAACCGAAN GCNCGCAAAC NAAGGTTCTT CGGGAAAAAA       600
40
         AATCGCCAAA ATACNCGANA TCAAACTNTC CAA                                   633
```


(2) INFORMATION FOR SEQ ID NO:22:
45
            (i) SEQUENCE CHARACTERISTICS:
                  (A) LENGTH: 213 base pairs
                  (B) TYPE: nucleic acid

(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```
TGCCGAGCTG GGGGGAGTTC CAGGAATTTG TGGACTATTT CCAGGAGGAA TTGAGGAATC        60

TAGAAGTAAT AAGAACTTCA CAAGTAGAAC AACAGAGTTA ATTGACCTCT ATCCTTAAGA       120

GTTACCAGAG AATTATTAAA AAACTAAAGA ACAATCAAAG CCTGGTCCTG TGCCACCACC       180

CAAAAACATG TATAGCCTAT GTGCAGCTCG GCA                                    213
```


(2) INFORMATION FOR SEQ ID NO:23:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 679 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```
CTCANAGGGC NNNTTNGNGG NCNTCATGCN CCAGGNTCCN NCCCCCANAN GANCNNCCNG        60

GTAAACTACA CNGGAGTACT TAAGTGGACA NNCCACATGC GANGGNCAAG GGGATCACCN       120

TCNCTCCTNC AGNCTNTNCG TGNCTCTCCT GTNCNTNCAC TGCCNCANAA NGGANGCNCN       180

NNCTCCTATC TGTNTACAGN AAACNTNGCN CTNNCTCTAA GCTCNCCCAC TNTGTGGAAA       240

GGCNATGTGT GCGTGCCTCT CCCCTATCAC GGCNGTTTGC NAAANGGGGA TGTNCTGCNC       300

GGCGATGAAG TTNGGTCACT CCATGTTTCC CAGTCCNACC TGTTAGACNA AGNATTGNAN       360

TGTGATACGA CTCNCTGTAA GGGGANTNGC GGACCCAGTA TGTTTGGCCC NACNNCCACT       420

TCTTTAAATG GTGGCTAACG GCGCTTCCTA GNATAAACAC TATTGGTCCC CCCCTCTGCA       480

GNACCCNTTA CTTCCGNANA AAAATTGTTG TCNTGATCCG CGACAACCAC ACCGTCTGTN       540
```

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```
GNTTTTAGTT GCAACNCNNA TCNCTCCAAA AAAGTTTCAG AAATCTTCAT TTTCCCNGGT       600

TGAGCCCNTG ACAAACCCCT NAGGATTTGT CGAATGTAAA GTCTCCNGAT CTTCAATAAA       660

NNTCCAAAAG NCTANCGAT                                                    679
```

(2) INFORMATION FOR SEQ ID NO:24:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 717 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

```
NTCNNCTTNN CNNNGGCTGA TATCNGGCNC TTCNTCCNCG ATCNCAGATA CNNGCNCACC       60

GGNNNTNTCN GNGGTNATCN TCCNCCATCT CTCNTCCCCG ACNTGCACTC CGGGTNTNNT       120

ACACNGGACA CTGTATCNNA CAGNAAACCT NCCCNGGCCC CAGGGATCAC CATNCCTCGN       180

CCCNGCNTGT NTATAANATC AGGNNNTACA TCNANGAACN NACTATCACN GNTCTCTNTT       240

NNCTCAGTGT NCACCTTCCA CTNCNGAANC TNNTCGCTNC NCCNCNGTTG GGAAAGGCGA       300

NCNGTNCCGG CNACATGCCG TTTNCGNCNT CTGNNCACNT GGGGATCTNC TNCAANGNAA       360

TCAATTNGNG TAACCCACGG TTTNCNCAAT CACTACTTCT CANNCNANGG CCNTTGAANT       420

GTTATCCCAC CACCANGGGG CNANTCGGGA CCTNACAATT CATCCTCAGC CGGCCCCAGN       480

CTTAAAAAAT TCAAAGGNCN CTTGCCCGCN TTNTTNCCTT AGCCCGCCNC CNGACAACAN       540

CCNANNAACA ACCCCCNNTC TTANGTTGCN NANCCCACAG GANNTTGNNA TACCGGGTTT       600

CCCCNGAAAC TNCTCAANGC CNCCGTTCCA ACCCCCGTTA CGAAACCGTN CCCNTTTCCT       660

TCCGAGNTTG CCTATTAANN CCCCCNAAGT TCTNCTTCGT TNGNTTCCTC CGAAANG         717
```

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/04323

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12P 21/02; C12Q 1/68; C12N 15/11
US CL : 530/350; 536/23.1; 435/6

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 536/23.1; 435/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4,981,783 A (AUGENLICHT) 01 January 1991, col. 2, lines 40-64.	19
A	US 5,599,672 A (LIANG et al.) 04 February 1997, see entire document.	1-40

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
02 JUNE 1999	15 JUN 1999

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	JAMES KETTER
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INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 20-40
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

   The Computer Readable Form (CRF) of the Sequence Listing as filed does not comply with 37 CFR § 1.821-1.824. A; such, claims 20-40 could only be searched in part, by word searching.

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

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